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14. ABSTRACT

The factors contributing to heterotopic ossification (HO), the formation of bone in abnormal soft-tissue locations, are beginning to emerge, but very little is known about the microenvironmental conditions that promote this often devastating disease. Using a murine model in which endochondral bone formation is triggered in muscle by bone morphogenetic protein 2 (BMP2), we studied the changes near the site of injection of BMP2-expressing cells. We propose to study in more detail the role of adipocytes in heterotopic bone formation in order to prevent or treat heterotopic ossification. We will augment our current microarray analysis and immunohistochemical analysis of the kinetics of HO. We will compare, using histology, immunohistochemistry, and micro CT the mouse and human lesions. We will also address the reason that combat wounds are more likely than civilian trauma to develop heterotopic ossification. We hypothesize that there is a basic difference in the injury pattern/mechanism that may impact nerves, recapitulating spinal cord injury in which HO is a major problem. We therefore also plan to study the neuronal component of HO. Finally, it is important that in blocking HO we do not also block the normal fracture repair process; this latter process should be intact or even augmented.

15. SUBJECT TERMS
BMP2, heterotopic bone formation, heterotopic ossification, adipocytes, fat metabolism, stem cells

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Introduction:

Heterotopic ossification, defined as the formation of bone in abnormal anatomic locations, can be clinically insignificant or devastating, depending on the site and duration of new bone formation. There are many causes of heterotopic ossification, including soft-tissue trauma, central nervous system injury, vasculopathies, arthopathies, and inheritance. Fibrodysplasia ossificans progressivia (FOP) is a rare genetic disorder in which disabling ectopic ossification progresses in a typical anatomic pattern until most or all major joints of the axial and appendicular skeleton are affected. Arterial ossification and cardiac valve ossification appear to be highly regulated processes, possibly mediated by bone morphogenetic proteins (BMPs).

Attempts to prevent or treat heterotopic ossification have been restricted by the complexity and multiple causes of the disorder. Nonetheless, new therapies are being devised targeting the inductive molecules that may trigger the process, participating progenitor cells, and local tissue environments conducive to osteogenesis. Gene therapy with BMP antagonists seems especially promising because overexpression of BMP4 and underexpression of physiologic BMP antagonists are common findings in some forms of heterotopic ossification. Because angiogenesis is absolutely required for endochondral bone formation and is a prominent histopathologic feature of the preosseous lesions in FOP, targeting new blood vessel formation with antiangiogenic agents may slow or inhibit the production of heterotopic bone.

To gain a more complete understanding of the factors that drive heterotopic ossification, we focused on the microenvironmental conditions needed to induce mesenchymal stem cells to differentiate to chondrocytes, which form the cartilagenous matrix essential to osteoblast recruitment and normal osteoid mineralization during endochondral bone formation. Several studies suggest that low oxygen tension critically influences chondrocyte differentiation by accelerating the growth of mesenchymal stem cells and promoting their commitment to the chondrocyte lineage, in part by upregulating a program of chondrocyte-specific gene expression under the control of hypoxia-inducible factor 1 (HIF-1). Although the requirement for low oxygen tension during the initial stages of endochondral bone formation is well accepted, the source of hypoxia in local tissue environments remains largely undefined. To address this issue, we relied on a model of heterotopic ossification in which human fibroblasts are transduced to express BMP2 and are then injected into a hind-leg muscle of mice. BMP2 has been used extensively to induce bone formation in patients and differs from BMP4 by only a single amino acid change (valine instead of alanine at position 152), Preliminary analysis of the tissue changes within the lesional area suggested that BMP2 released by the transduced fibroblasts recruits and stimulates mesenchymal elements to form mature marrow-containing bone. This model appears highly relevant to ectopic bone formation in humans because it uses a BMP that is virtually identical to BMP4 to stimulate endochondral bone formation in extraskeletal muscle. Indeed, both loss-of-function and gain-of-function studies have demonstrated the necessity and sufficiency of BMP2 and BMP4 in regulating the development of cartilage and bone.

Body:

Task 1: Develop vectors for preclinical testing:

A. Constructing vectors necessary for testing:

Most of the vectors as outlined in the application have already been constructed including the Ad5noggin and Ad5BMP2 vectors and all controls. **However, we are asking for previously asked for permission to vary from this approach. First, since delivery of noggin will inhibit the BMP2 only if it is present at the earliest onset, we have requested the ability to try to develop a method for early detection of heterotopic ossification, so that we could locally deliver the vector at the appropriate time. However, the targets that appear to be most feasible to localize HO prior to matrix deposition, are events that are induced by the BMP2, hence, delivery of noggin at this point would be ineffective. Thus, we have requested the ability to target some of the factors just slightly down stream of the BMP2 receptor signaling. Secondly, we request permission to terminate the work with the vectors which regulate adipocyte regulation.** We have used both these vectors as well as a number of different adipose knock out animals, including PPAR alpha$^{-/-}$and Brown adipose$^{-/-}$ and found that inhibition of brown adipose does not seem to effectively block HO, but rather at times enhance the process. For both the knock out animal models, we observed substantial new bone formation. Interestingly, however, the bone in the PPAR alpha$^{-/-}$ model, showed a substantial change in the marrow cavity. As can be seen in figure 1, the internal cavity of the HO, which usually has a large amount of white adipose, now has edema, and blood, suggesting that we have altered perhaps the vasculature, as well as the adipose, and resulting in similar or more bone (figure 2) but without normal soft tissues, which would potentially be a more detrimental process. **To this end, we request permission to focus on the two the stages, first the extravasation step, necessary for providing progenitors, and the earliest stages involving the peripheral nervous system. Thus please see below for more details.**

B. Define novel molecular targets in early heterotopic ossification to block the process.

We have recently described that brown fat is a key intermediate in bone formation ([1], one purpose is to create an hypoxic environment for chondrogenesis. We have now determined that the stem cells for bone formation, including progenitors for adipocytes, chondrocytes, osteoblasts, osteoclasts, originate in bone marrow and are delivered to the site of bone formation by new vessels that are composed of sinusoidal endothelial cells. We have also identified key components in the extravasation of these stem cells through the vessel to the site of heterotopic bone formation. These include Selectin E, CD44, SDF1, CXCR4, CD11b, and fucosyl transferase 8 (Table 1).

Table 1 shows the top 23 genes that are elevated during this time period. The highlighted genes have been shown to function in the transport of circulating cells across the vessel to targeted sites

We have characterized the expression of these genes within the tissues, with the peak of expression occurring between day 3 and 4 after induction of HO with BMP2, and completely absent in the matched controls (Figure 3). Figure 1 depicts the current model for mesencymal stem cell extravasation through the vessel into either injured tissues or their “niche”. Immunohistochemical analysis demonstrates strong expression of markers for every stage of this process. Interestingly, we do not see these expressed in the control tissues even though

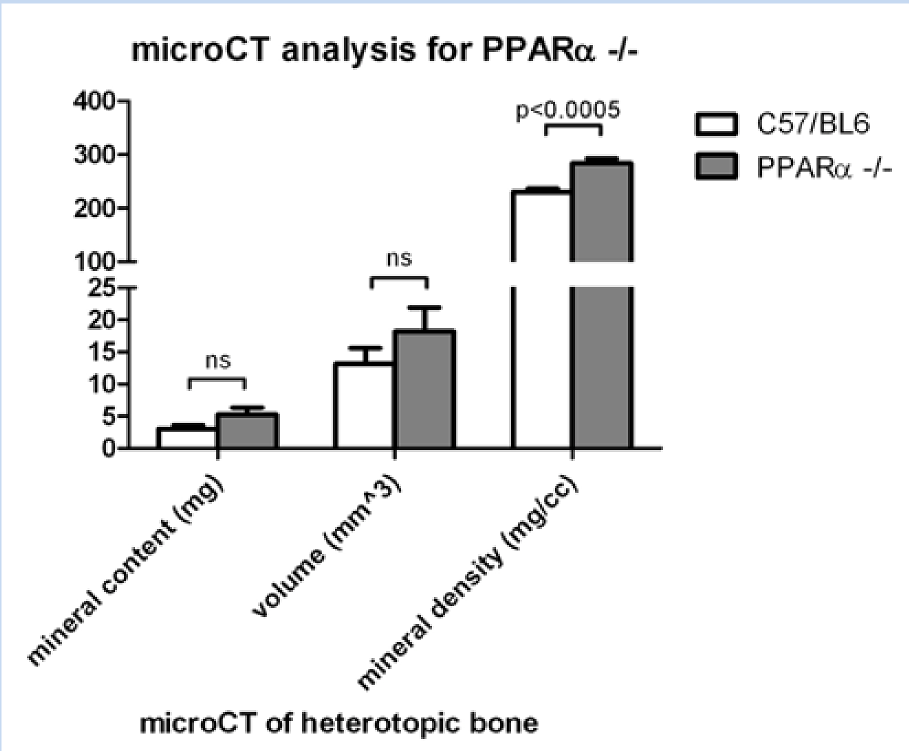


Figure 2: Quantification of heterotopic bone formation through microCT analysis.

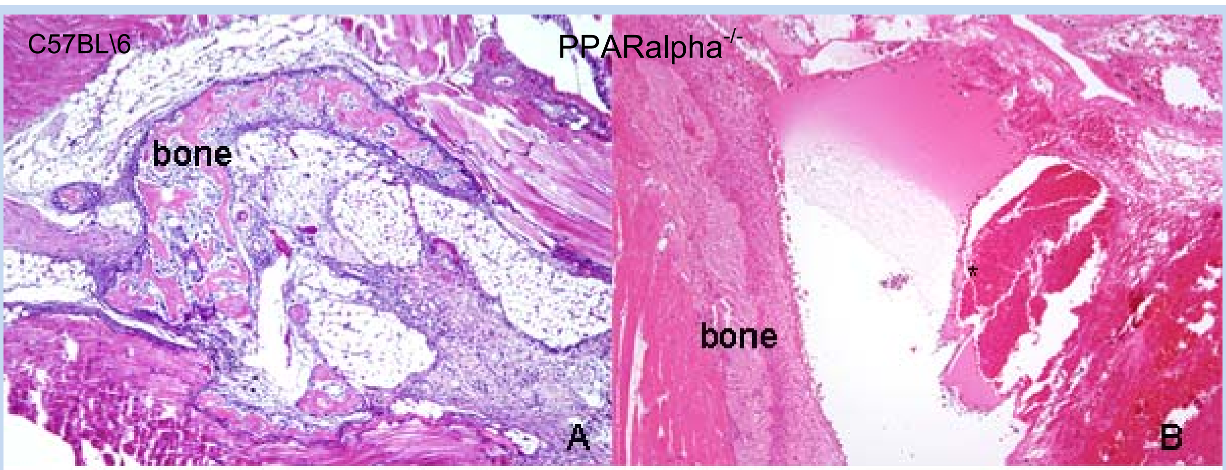


Figure 1: Photomicrographs of heterotopic bone formation in either C57BL\6 wild type mice or PPARalpha knock out mice which have significant defects in adipogenesis and adipose.

Table 1. Genes upregulated on the third day after injection of BMP2-producing cells. Highlighted gene products are part of the pathway of stem cells through the vessel.

Gene Name	Relative Expression
Cox-2	2478
Stearoyl-Co Enzyme A desaturase 1	2085
Selectin E	1641
Apolipoprotein E	1613
CD44	1060
SDF-1	877
Solute carrier family 2	773
Protein Kinase C gamma	659
Aryl hydrocarbon receptor	625
Decorin	603
SMAD 3	595
Protein Kinase C epsilon	547
CXCR4	447
Myosin 1B	438
S-phase kinase-associated protein	396
Mac-1 (CD11b)	328
B-cell receptor associated protein 37	25
Enolase 3	24
Fibulin 2	23
Fucosyltransferase 8	23
EGL nine homolog	22
Dynein cytoplasmic heavy chain 1	19
Adaptor-related protein complex 1	17

inflammatory processes occurring in the control may result in lymphocyte extravasation. Presumably this is due in part to the use of different family members in for extravasating lymphocytes versus mesenchymal stem cells. Since entry of progenitors into the site of HO induction is the first step in creating the bone, this would be an ideal step to target through inhibitors. Further, because these family members appear to be specific to the stem cells, select agents that would more specifically target these members could greatly reduce potential negative side effects from delivery of the inhibitor. Since there are several inhibitors of SDF-1 and CXCR4 binding currently under development, **we propose this to be a great site to initially target.** To complete our analysis of this work, we are confirming the microarray analysis and immunohistochemistry with quantitative PCR against these selected genes.

We have isolated RNA across each day of the time course, and we will quantitatively measure the expression of these factors as well as other family members known to be involved in either leukocyte or stem cell extravasation. As can be seen in Figure 4, results of the q-RT-PCR show a statistically significant elevation in CD44, E-Selectin, P-Selectin, and CXCR4 however we did not observe statistically significant changes in SDF alpha, and are currently following that up (figure 4). We also looked at the expression of the selectin proteins in the tissues. And found that there was also a significant elevation in protein levels as well as the

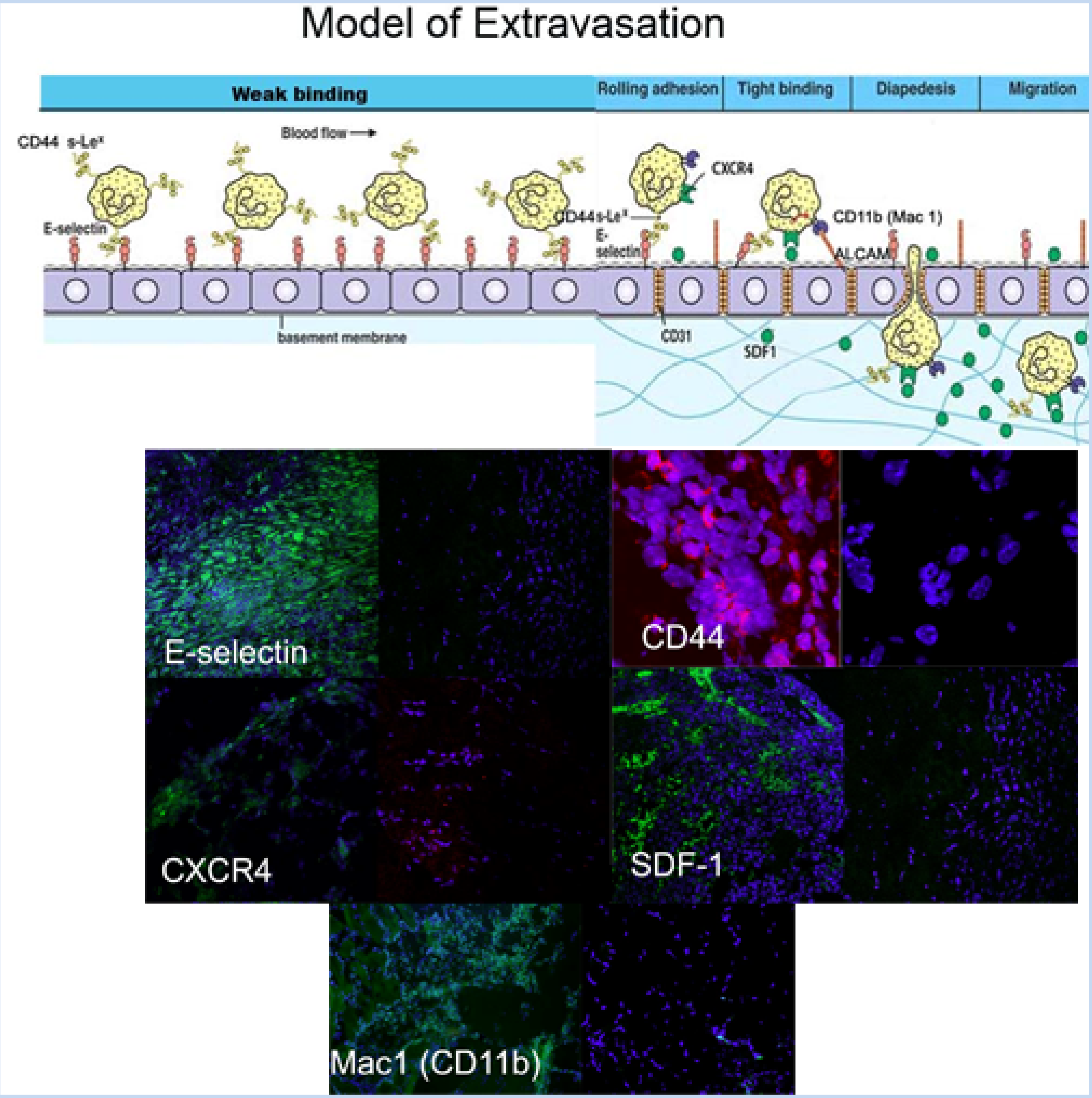
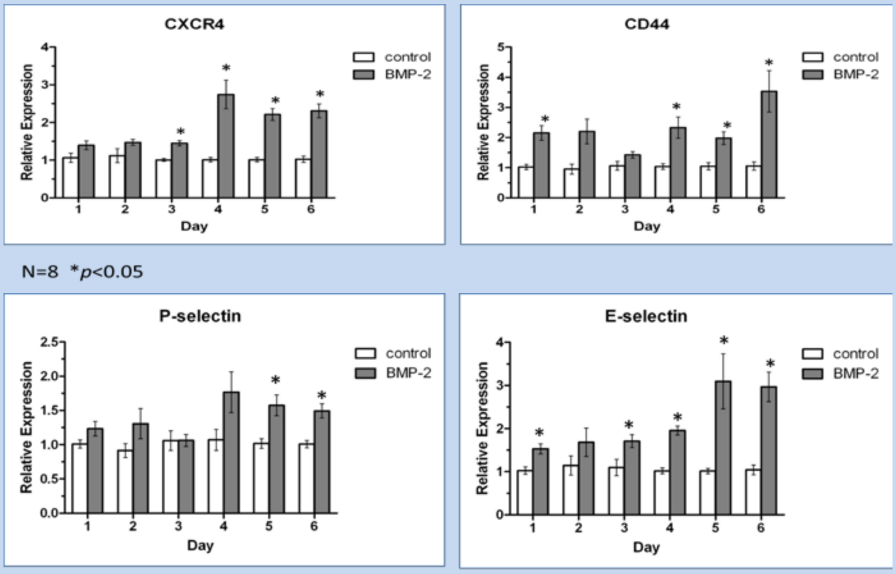


Figure 3: Model of Extravasation. Immunohistochemical analysis of stem cell extravasation in tissues isolated three days after induction of heterotopic ossification by delivery of either cells transduced to express BMP2 of in the case of the control tissues cells transduced with en empty cassette vector. Stem cells are thought to enter tissues through a process known as extravasation. This involves the expression of key factors which bind between the vessel wall, and the stem cells. Two binding pairs are CD44 - Selectin (E or P) and SDFalpha – CXCR4 which are known to be essential to this process, and capable of regulating stem cell entry.

RNA. As can be seen in Figure 5, both E- and P- selectin known to bind CD44 on the surface of stem cells, was more highly elevated in the BMP2 tissues as compared to control. Interestingly, the E- selectin was elevated 48 hours after delivery of the AdBMP2 transduced cells, while the P-Selectin appear to be elevated

As seen in the next section, the new vessels appeared within 48 hours, as well, suggesting that perhaps the new vessels are being formed to carry out extravasation of osteoprogenitors into newly formed calcified cartilage. We are actually working on assembling this manuscript which should be completed in by the next quarter.



N=8 *p<0.05

Figure 4: Results of the quantitative RT-PCR for extravasation factors during formation of heterotopic bone. The results are a comparions on control tissue or those which received Adempty cassette transduced cells, versus those receiving AdBMP2 transduced cells

c. New vessel formation and fat in heterotopic bone formation.

We recently published work which demonstrated the rapid formation of new vessels prior to the appearance of cartilage [2]. The timing of the new vessels in association with the expression of the extravasation markers suggests that this process is essential for recruiting progenitors to the site of bone formation. Thus we are current working on demonstrating the presence of bone marrow derived or circulating stem cells, for cartilage and bone, and that these cells can be effectively blocked using the CXCR4 inhibitor AMD3100. AMD3100 binds to CXCR4 and prevents binding to SDFalpha and entry into the tissues. The drug is currently on the market and is considered to have few associated adverse reactions associated with it.

Therefore to clearly demonstrate the recruitment of circulating stem cells, and contribution to heterotopic ossification, we presently have a series of mice which have been repopulated with HSCs (ckit+, Sca1+, Hoescht dye low, tie 2+) possessing the GFP reporter, two groups are being assessed one, receiving AMD3100, and the other receiving PBS, prior to receiving the AdBMP2 or Adempty cassette transduced cells. Then, 2-10 days after induction of HO, tissues were isolated and imaged for GFP positive chondrocytes and osteoblasts. Unfortunately we did not find the reporter prevalent in the new bone formation suggesting that there may be another source for stem cells. Recently Medici *et al* [3]showed the contribution of a local vascular progenitor, to the bone and cartilage. However our own work shows that essential progenitors for the bone formation may be derived from peripheral nerves [4].

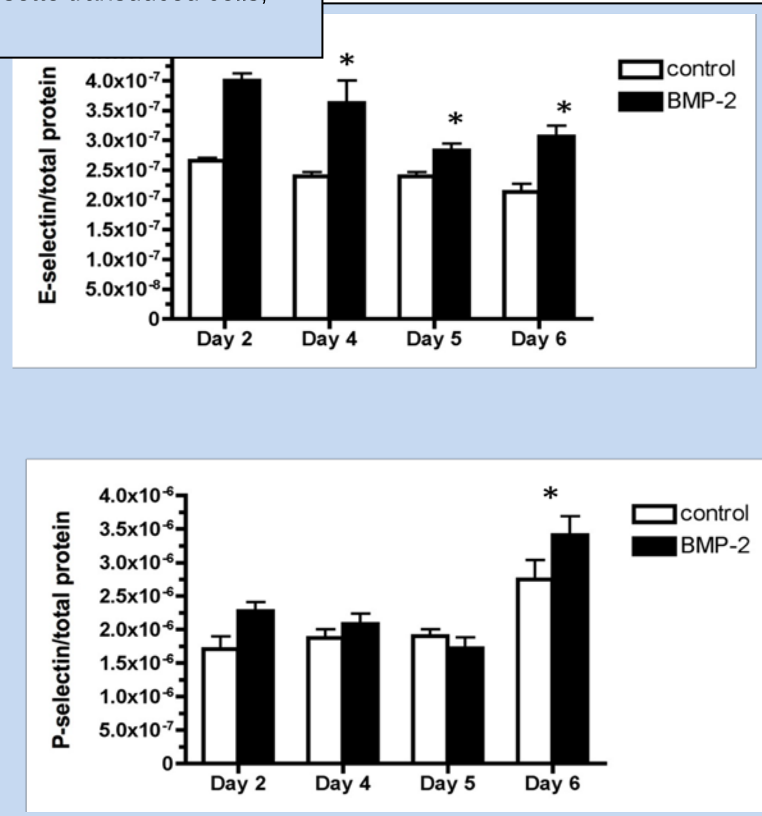


Figure 5: Quantification of E and P selectin in tissues after delivery of AdBMP2 or Adempty cassette transduced celsl. The proteins were quantified by ELISA. Levels of E - and P- Selectin are both elevated after induction of heterotopic ossification, however at different time's post- induction. E-selectin, was highly elevated within 48 hours after delivery of AdBMP2 transduced cells, as compared to the control and remained elevated at all time points.

cartilage. However our own work shows that essential progenitors for the bone formation may be derived from peripheral nerves [4].

d. Heterotopic bone and peripheral nerves

Our studies suggest that heterotopic ossification (HO) [5 6 7] and the endochondral bone formation follows an ordered series of steps involving a number of soft tissues prior to the deposition of matrix (4)[8] which first appears in this model by 6-7 days after initial induction. Microarray analysis performed at daily intervals across the course of this process, suggests immediate changes in peripheral nerves (PN) adjacent to the site of HO. Since heterotopic ossification appears to have a strong correlation with traumatic injury (CNS injury, myositis ossificans, hip replacement), and specifically the types of injury that would affect peripheral nerve signaling, or with diseases of HO such as (fibrodysplasia ossificans progressiva (FOP). Further it is intriging that recent reports from the military suggest that although the incidence of HO associated with traumatic injury in a civilian population is approximately 10%; in the military population it is 60%, suggesting that the types of bomb and blast injuries are capable of triggering the PNS stimulus which induces HO.

BMPs appear to play an essential role in nerve cell differentiation in the embryo (see [9] for review), but studies using dorsal root ganglia cultures showed that BMPs were capable of directly stimulating release of substance P and CGRP from sensory nerves [10]. Reports in the literature show a direct role for BMP2 in release of neuroinflammatory molecules, suggesting a potential similar role in vivo during HO formation. This led us to hypothesize that BMP2 may be directly activating the peripheral nerve to release the neuroinflammatory factors, substance P and CGRP. Release of these factors has been shown in the literature to recruit mast cells directly to the nerve. This migration eventually leads to degranulation of mast cells, which have been hypothesized to produce several enzymes involved in remodeling and degradation (Figure 7). To determine if this mechanism was a component of the BMP2 mediated HO in our model, we have immunostained for the presence of both CGRP and substance P in our tissues and found substantial positive expression in animals undergoing HO as compared to the controls (Figure 8). As can be seen in Figure 8, we observed significant positive staining associated with the nerve in tissues 48 hours after induction of BMP2, as compared to the tissues receiving the control cells.

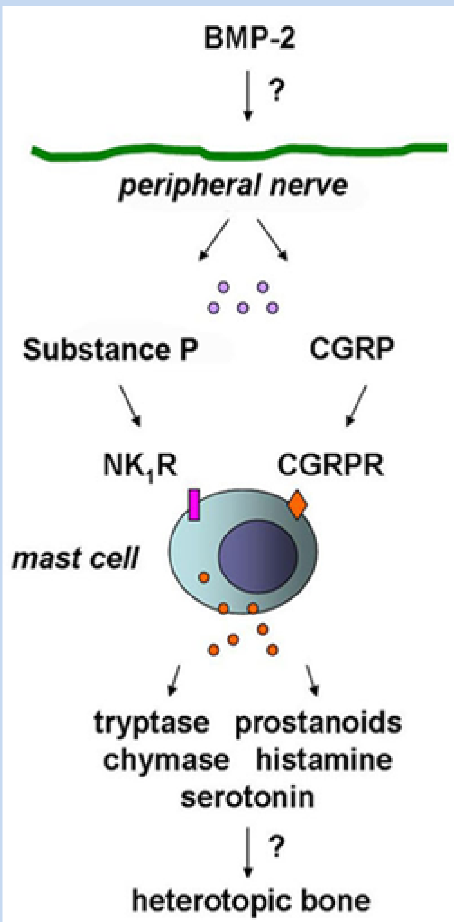


Figure 7: Schematic of mechanism of BMP2 action in HO formation, based on *in vitro* studies in the literature.

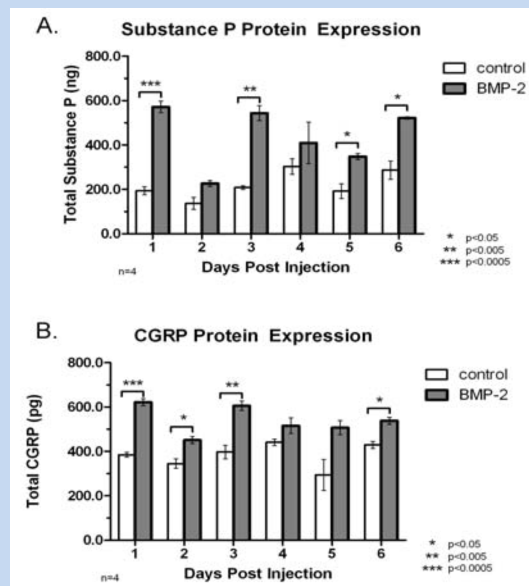


Figure 8: Quantification of substance P and CGRP protein by ELISA. Soft tissues, which encompass the site of new bone formation, were isolated at daily intervals from animals receiving either AdBMP2 (BMP2) or Adempty (control) transduced cells, and protein extracts were generated. **A.)** Substance P total protein was quantified and statistically significant changes between the groups, as denoted by an asterisk, determined using a standard t-test; n=4. **B.)** CGRP total protein was quantified and statistically significant changes between the groups, as denoted by an asterisk, determined using a standard t-test; n=4. * denotes statistical significance.

To determine if BMP2 directly activates expression of the neuroinflammatory proteins SP and CGRP during heterotopic ossification, proteins were isolated from tissues at daily intervals, starting 24 hours after delivery of AdBMP2 or Adempty (control virus) transduced cells, through the appearance of heterotopic bone. Quantification of protein levels of SP and CGRP within the tissues, through ELISA, is shown in figure 1A and B, respectively. Both proteins appear to be significantly elevated ($p \leq 0.0005$), compared to controls, within 24 hours after induction of HO, and again at 72 hours ($p \leq 0.005$) and 6 days ($p \leq 0.05$) after induction. Expression, therefore, appeared somewhat cyclical, and statistical analyses, using a one-way ANOVA with a post-hoc Bonferroni test for comparison between time points, verified a significant drop in SP and CGRP between days 1 and 2 ($p \leq 0.005$). This was followed by a significant rise between days 2 and 3 ($p \leq 0.005$). The data suggests that BMP2 induced a substantial and immediate release of these proteins, which was attenuated, but then continued for the remainder of endochondral bone formation, through the appearance of mineralized bone (Figure 8).

Tissues were next immunostained for the presence of SP and CGRP and analyzed to determine if the

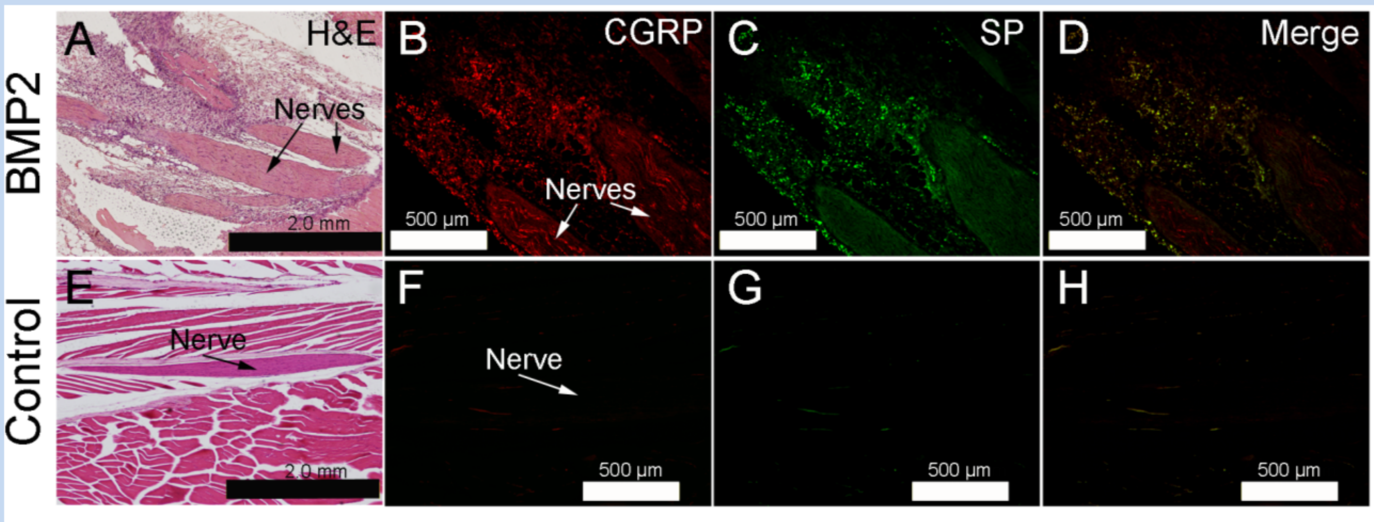


Figure 9: Photomicrographs of substance P and CGRP protein expression in tissues isolated four days after induction of HO. Tissues receiving cells transduced with AdBMP2 (BMP2) or Adempty cassette (control) were isolated four days after induction and immunostained with antibodies against substance P and CGRP. Expression of these factors was found to co-localize with the area of newly forming bone, but was minimal in tissues receiving the Adempty cassette transduced cells. Hematoxylin and eosin stained serial sections, adjacent to the section used for immunostaining four days after receiving **(A)** AdBMP2 transduced cells or **(D)** Adempty cassette transduced cells. Positive staining in the region of new bone (**B and C**) or nerve (**E and F**) for CGRP (B and E; red color) or substance P (C and F; green color).

expression of these factors was associated with nerves throughout the entire hind limb, or limited to the region of new bone formation. Figure 2 shows representative images of the expression of SP and CGRP within the tissues isolated 4 days after receiving either AdBMP2 or Adempty transduced cells. By day 4 of HO, delivery cells are no longer found within the tissues, and the control appears as normal muscle (Figure 9D). However, there is a nidus of cartilage forming in tissues undergoing HO (Figure 9A). Figure 9 shows the positive expression of SP (green) or CGRP (red) in these tissues. As seen in figure 9, panels D-F, we observed a small amount of positive expression associated with a mature nerve structure within control tissues, but expression was not found within the muscle itself. In contrast, in tissue receiving BMP2, expression was co-localized with the region undergoing cartilage formation and was limited to this region. This suggests that the expression of these factors is associated with BMP2, as predicted, and the continued expression of these factors was localized to new cartilage and bone formation.

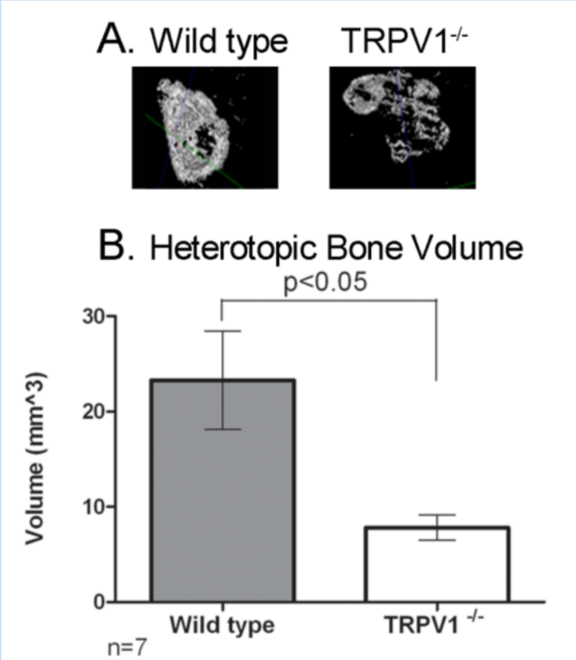


Figure 10: Microcomputational analysis of heterotopic ossification ten days after induction with AdBMP2 transduced cells, in C57/BL6, wild type or TRVP1$^{-/-}$ mice. **(A)** Three dimensional reconstructions of representative samples for each group. **(B)** Quantitation of bone volume and statistically significant changes between the groups was determined using a one-way analysis of variance; n=7. * denotes statistical

The induction of neuroinflammatory mediators occurs through activation of sensory neurons by localized stimulus, or, in this case, secretion of BMP2. To determine if induction of neuroinflammation is contributing to HO, bone formation was quantified in animals that lacked TRPV1 (TRVP1$^{-/-}$), resulting in a functional loss of activity of sensory neurons. These TRVP1$^{-/-}$ animals lack a functional cationic channel on peripheral, sensory nerve terminals, which regulate neurogenic inflammation. We quantified the changes in SP and CGRP protein expression within tissues isolated from these knockout animals, and observed a significant suppression compared to the wild type counterpart [4] although we did observe a slight increase in their expression upon delivery of BMP2.

HO was induced in both TRVP1$^{-/-}$ and wild type mice (n=7), and, after 10 days, the resultant bone formation was quantified through micro-computed tomography (µCT). Figure 10A shows a representative three dimensional reconstruction of the bone formation. Heterotopic bone volume within TRVP1$^{-/-}$ mice was inhibited significantly ($p \leq 0.05$), as compared to wild type mice (Figure 10B).

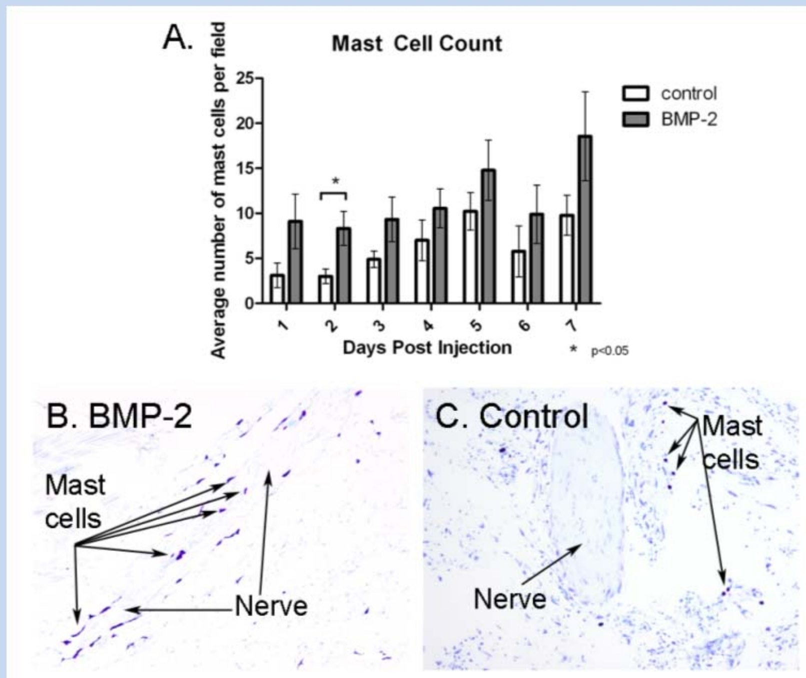


Figure 11: Quantitation of mast cells in tissues surrounding the area of new bone formation. Tissue sections were stained with the mast cell stain, toluidine blue. Photomicrographs of random fields (5 per section) were taken at 10X magnification, for every fifth slide, throughout the entire hindlimb. Each field equals 5.2mm^2. **(A)** Quantification of the average number of mast cells within the tissues at daily intervals after induction of HO. Statistically significant changes were determined using a standard t-test; n=3 biological replicates. Seven slides were analyzed per tissue, and 5 fields per slide (35 images quantified per sample/time point). * denotes statistical significance. **(B)** Representative photomicrographs of tissues isolated two days after receiving AdBMP2 (BMP2) or Ademptу (control) transduced cells, stained with toluidine blue. Positive cells are highlighted with arrows.

The reduction of HO when there is a lack of functional TRVP1 signaling suggests that this pathway may be functionally important to the process of HO. The next step in neuroinflammatory signaling involves recruitment of mast cells and their resultant degranulation, for the release of key enzymes involved in processing proteins essential for inflammatory signaling and recruitment. To determine whether mast cells were recruited to the site of new bone formation, muscle tissues from the hind limbs of wild type mice injected with AdBMP2 or Adempty transduced cells were isolated at daily intervals and then serially sectioned in entirety for quantification. There appears to be a trend toward more mast cells within the tissues undergoing HO, as compared to the control tissues (figure 11A). However, only day 2 shows a statistically significant increase in the number of mast cells. It is intriguing that we observed the most significant difference at these early stages, since this appears to parallel our findings for the release of SP and CGRP within the tissues, suggesting mast cells may be recruited after release of these factors. Since mast cells are known to migrate throughout the tissues, co- localization with specific tissue structures was also noted. As seen in figure 11C, mast cells appeared to be scattered throughout the control tissues. However, within the tissues receiving AdBMP2 transduced cells, mast cells associated only with the nerves (figure 11B), in tissues isolated 2 days after induction of bone formation. As bone formation continues, the mast cells within the tissues receiving BMP2 continue to be localized within the nerve itself; however, a subset also appear within the vessel structures (data not shown). We did not see mast cells localizing within the nerve structures in control tissues at any time point.

Mast cell degranulation leads to the release of degradative enzymes, such as tryptase and chymase. These enzymes are known to degrade or process other proteins, leading to their activation. Many of the enzymes are involved in tissue remodeling, including the nerve structure itself. To determine if mast cell degranulation could be a factor in heterotopic ossification, animals were pretreated with the drug sodium cromoglycate (cromolyn), which has been shown to prevent mast cell degranulation. Following the pretreatment with either cromolyn or a vehicle control (PBS), HO was induced and the resultant bone formation quantified 10 days later. Figure 12A shows representative images of three dimensional reconstructions of the resultant HO formation after cromolyn or control, PBS treatment. As can be seen in figure 12B, quantification of bone volume of the HO shows a significant ($p \le 0.05$) decrease in animals after cromolyn treatment. The observed decrease in HO formation after cromolyn treatment was similar to that observed in the TRVP1 null mice, thus supporting the idea that suppression of this pathway inhibits HO.

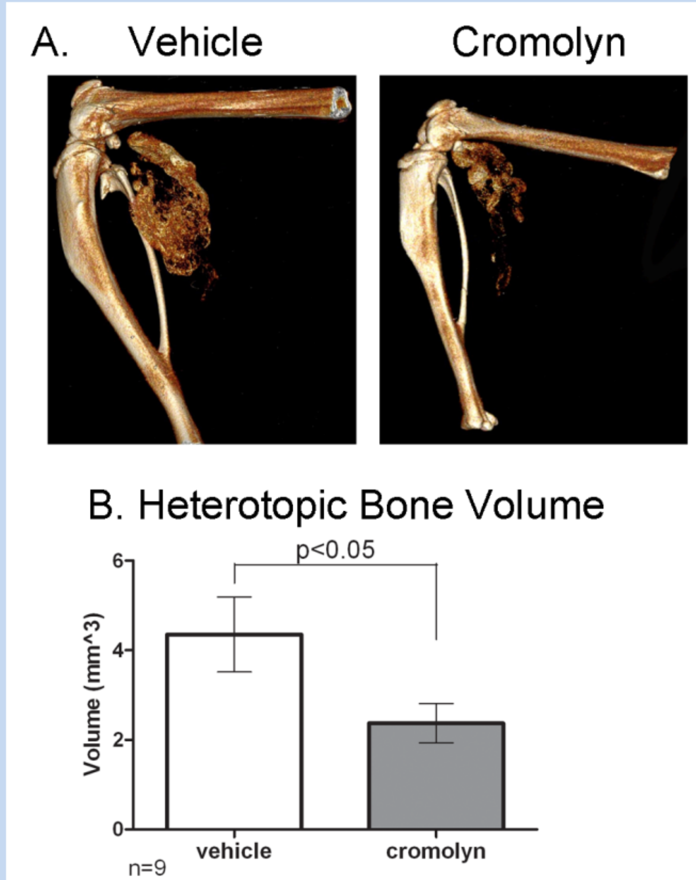


Figure 12: Microcomputational analysis of heterotopic ossification ten days after induction with AdBMP2 transduced cells, in mice pre-treated with either cromolyn or vehicle control (PBS). **(A)** Three dimensional reconstructions of representative samples for each group. **(B)** Quantitation of bone volume and statistically significant changes between the groups was determined using a one-way analysis of variance; n=9. * denotes statistical significance.

Mast cell degranulation is known to release enzymes essential for processing of other proteins. One key molecule mast cell chymase, has been demonstrated to process a protein known as CXCL7 to its active form. CXCL7 is actually made by platelets, and has been shown to be involved with recruitment of neutrophils. We next looked isolated some blood from animals 1 days after delivery of the AdBMP2 or

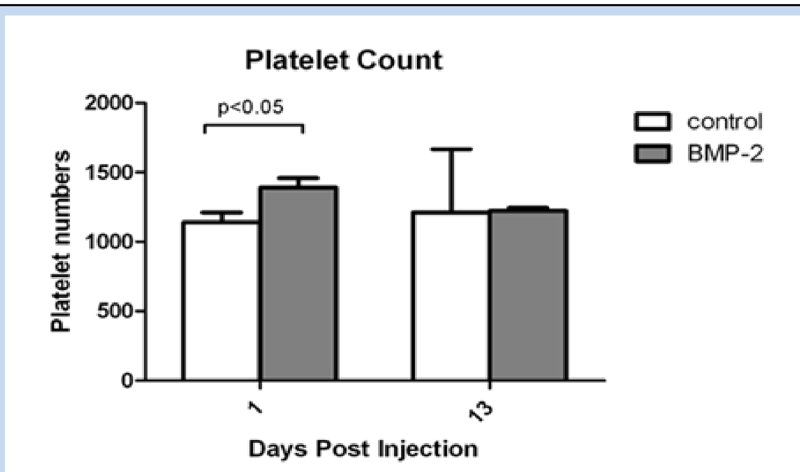


Figure 13: Elevation of Platelet Count. Blood was isolated animals on days 1 and 13 after induction of bone formation with either AdBMP2 or Adempty cassette transduced cells and sent to the Pathology Department of Comparative Medicine at BCM for analysis.

Adempty cassette transduced cells, and measured the number of platelets. The preliminary data is shown in figure 13. We observed a small but statistically significant change in blood platelets as early as 24 horus after induction of HO. However, by 13 days later, there was no difference in the level of platelets. We did not see changes in neurtrophils on day 1, but did observe a significant elevation at day 13. We have only looked at a small sample size of at the two time points, and if our model is correct we would expect the increase in neutrophils to be observed at 24-72 hours. We are currently focused on completing this analysis. **From this analysis we propose that changes in platelets and neurtrophil profiles in a simple blood test could provide a front line screen for the early phases of HO.**

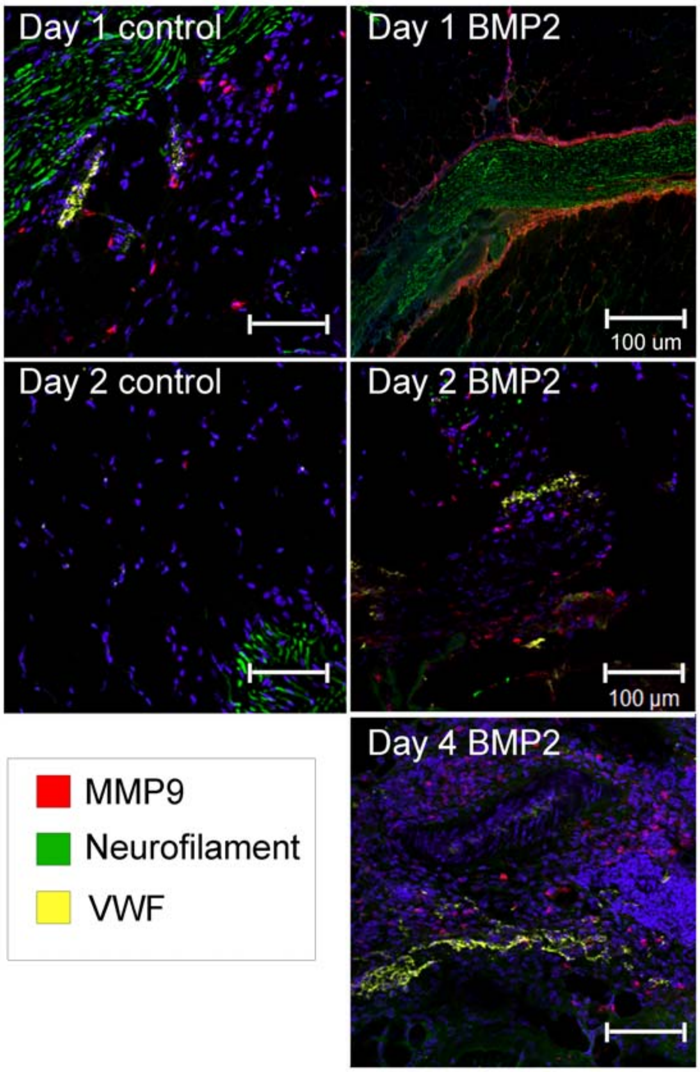


Figure 14: Photomicrographs of immunofluorescence staining for MMP-9 (red) neurofilament (green) and von Willibrand factor (VWF; yellow), in tissues following induction of HO by delivery of AdBMP2 or Adempty cassette transduced cells. Tissues isolated at daily intervals were serially sectioned, and every 5th slide stained and representative images are shown.

These platelets also express proteases that in turn process factors such as MMP9, that are involved with nerve remodeling, and release of nerve fibroblasts essential for neurite outgrowth. We analyzed the expression of MMP9 expression within the tissues.undergoing HO or the control using fluorescence microscopy. Interestingly, MMP-9 (red staining) was observed in tissues isolated 24 and 48 hours after delivery of AdBMP2 and Adempty transduced cells (figure 4). However by 72 hours, we were unable to detect MMP-9 positive cells within the tissues receiving Adempty (data not shown). MMP-9 expression (red) appeared to be associated with the nerve tissues (green staining) in the tissues one day after receiving AdBMP2 transduced cells, whereas MMP9 positive cells appeared to be uniformly dispersed within the control tissues (figure 14). In limbs injected with BMP2, at day two, we observed both nerve associated expression similar to day 1 (Figure 14), but also localization near von Willibrand factor (VWF) positive vasculature (yellow staining). This pattern continued through day four just prior to the appearance of cartilage (Figure14). Presumably this expression is associated with remodeling of the tissues at a point when progenitors are assembling to form the initial cartilage condensation.

To confirm that the elevation in MMP-9 RNA expression translated into enhanced protein expression in the tissues, MMP-9 protein was quantified by ELISA. Protein extracts were generated from the tissues isolated at daily intervals starting 48 hours after delivery of the transduced cells, and again ending at the onset of bone matrix production. Figure 15A shows changes in total MMP-9 protein over the course of heterotopic ossification. MMP-9 protein appears to be significantly ($p < 0.05$) elevated within 24 hours after delivery of the transduced cells regardless of the presence or absence of BMP2, but this elevation dropped rapidly, in the control tissues, whereas the tissues undergoing heterotopic ossification remained elevated. As seen in figure 15A, MMP-9 total protein was significantly higher in the BMP2-exposed tissues as compared to those receiving the control transduced cells with the exception of day 3. This may in part be due to the variability within the samples isolated from control tissues, and in part to the downward trend in total protein, after day 2. Interestingly, the level of MMP-9 protein trends upward again starting with day 4, attaining levels similar to those observed on day 2, at the time where cartilage matrix is undergoing degradation, and replacement with bone (day 6) (figure 15A).

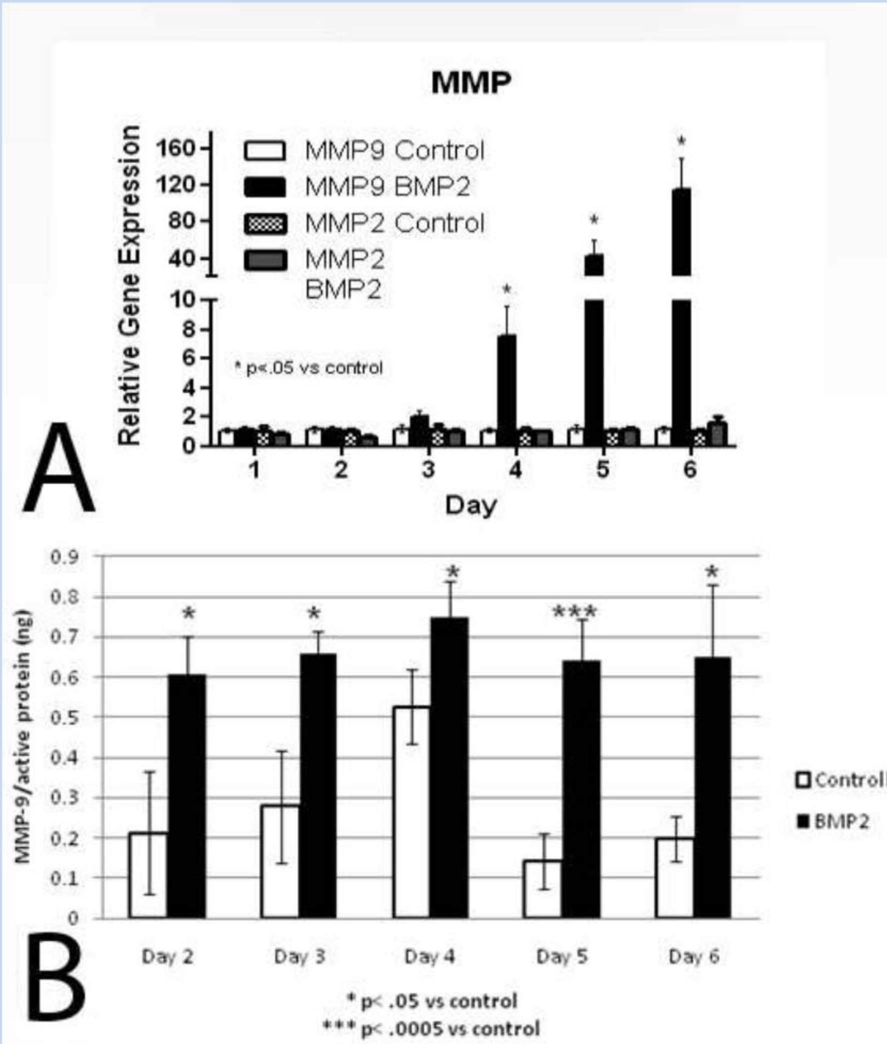


Figure 15A: Quantification of MMP-9 and MMP-2 RNA in tissues after delivery of AdBMP2 or Adempty cassette transduced cells. Total RNA was isolated and subject to quantitative Real-Time PCR. Each assay was performed in triplicate with n=8 biological replicates per time point. * denotes statistically significant change as determined by a standard T-test, in the sample from the control; p< 0.05

B. Quantification of MMP-9 protein and functional activity by ELISA. Tissues were isolated at daily intervals from animals receiving either AdBMP2 or Adempty cassette transduced cells and protein extracts generated. MMP-9 protein was bound through ELISA, and then substrate added, to quantify MMP-9 functional activity. Standard amounts of active MMP-9 were assayed in order to calculate active protein amount from the activity measurements. * denotes statistical significance.

Since the peptide reagent appears to preferentially detect active MMP-9, we next chose to measure amounts of MMP-9 using an ELISA-based system that detects only active MMP9. The results (Fig. 15B) show that protein extracts isolated from tissues receiving the BMP2-producing cells have significantly more active MMP-9 protein, than those receiving control cells and these elevated levels remained unchanged across the course of HO. The results collectively suggest that MMP-9 is activated by delivery of the BMP2-producing cells, during all stages of endochondral bone formation. Further, this activation may be due to cleavage and utilization of stored MMP-9 protein, immediately following induction of HO, but is then rapidly replaced by newly synthesized MMP-9. Activated MMP-2 remained below the level of detection (data not shown). The timing of MMP-9 expression within the tissue appears to match those predicted by the RNA and protein analysis.

We previously have shown a functional role for brown adipose in both induction of new vessels (both blood vasculature and lymphatic) Figure 16. Using a mouse which possessed the flk 1 promoter driving expression of the yellow fluorescence reporter YFP, we set up new bone formation, and measured the changes in YFP. We observed a significant elevation as early as 48 hours in these mice, suggesting that new vessel formation was occurring within the region of new bone formation even prior to cartilage. Further, analysis of the actual tissues figure 16 showed the presence of brown adipose expressing the vascular growth factor VEGFD adjacent to the flk-YFP positive small vessels. The data suggests that there is substantial new vessel formation which is coordinately regulated by transient brown adipose within the tissues.

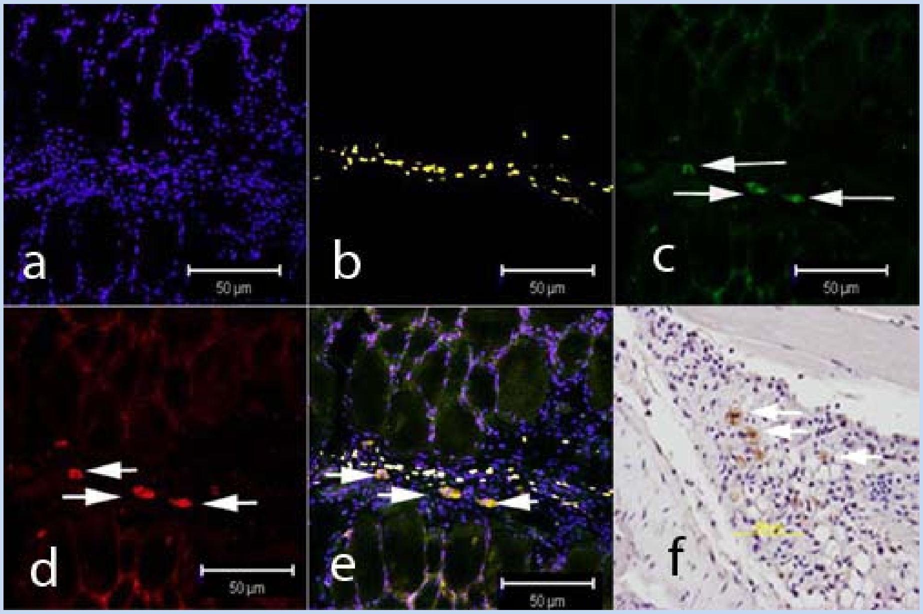


Figure 16: UCP1 positive brown adipocytes expressing VEGFD are found adjacent to new vessels.

Brown adipose plays a critical role in organizing the tissue undergoing *de novo* bone formation, and has been shown to be the product of sympathetic nervous system activity induced through the release of serotonin. Serotonin then binds its recepetor and leads to the release of noradrenalin. We next measured the levels of noradrenaline in the circulation of these animals after induction of new bone formation. Interestingly we saw a significant elevation in this factor within 48 hours after initiated bone formation, but this was transient (data not shown). Recent work in the literature [11] suggestst that the brown adipogenesis is induced through the activation of the β adrenergic receptors 1, 2 and 3, the receptors for noradrenaline. Our preliminary data (Fig 17) suggests that there is a strong correlation between β adrenergic receptor 3 (ADBR3) subtype and brown adipogenesis in our model of nerve remodeling and new bone formation. In these experiments cells positive for ADBR3, associated with the tissues surrounding the region of newly forming bone were sorted and immuno-stainned for UCP1 (fig1, pane A and B). There was no statistically significant increase in ADBR3 positive cells within these tissues until 3 days after induction of bone formation, with the peak being day 4 (fig 17 panel A). When these cells were sorted and immuno-stained for UCP1, there was a 100% correlation, whereas the ADBR3 negative population did not possess any positive staining for UCP1. Thus this marker correlates with the generation of brown adipose.

We next looked at the expression of ADBR3 within the peripheral nerves, and found that the majority was associated with the epineurial adipose regions of the nerve (data not shown). Interestingly, immediately (48 hrs) after induction of new bone formation, the majority of ADBR3 cells appeared to be associated as before with the epineural region, however, now there was the appearance of cells within the perineural space. Immuno-staining for UCP1 showed that the majority of the cells within the epineural region were negative for UCP1, only the perinerual cells positive for ADBR3 appeared to co-express the UCP1, suggesting that they may be derived from a progenitor housed wihtin the nerve sheath (fig 18). We further performed FACs analysis for ADBR3 positive cells from isolated sciatic nerves after induction of new bone formation and found a statistically significant increase in nerves isolated from animals treated with BMP2 within 48 hours, but a statistically significant decrease in this same population in nerves which were isolated 4 days after induction, suggesting that the cells associated with the nerve may have migrated into the tissues for new bone formation. Further support of this, is finding that there is no statistically significant difference in ADRB3 cell populations within the tissues surrounding the site of new bone, until 3 days post induction, with the peak at 4 days, suggesting that these cells may have migrated from the nerve to the new location within the region of new bone formation (fig 17).

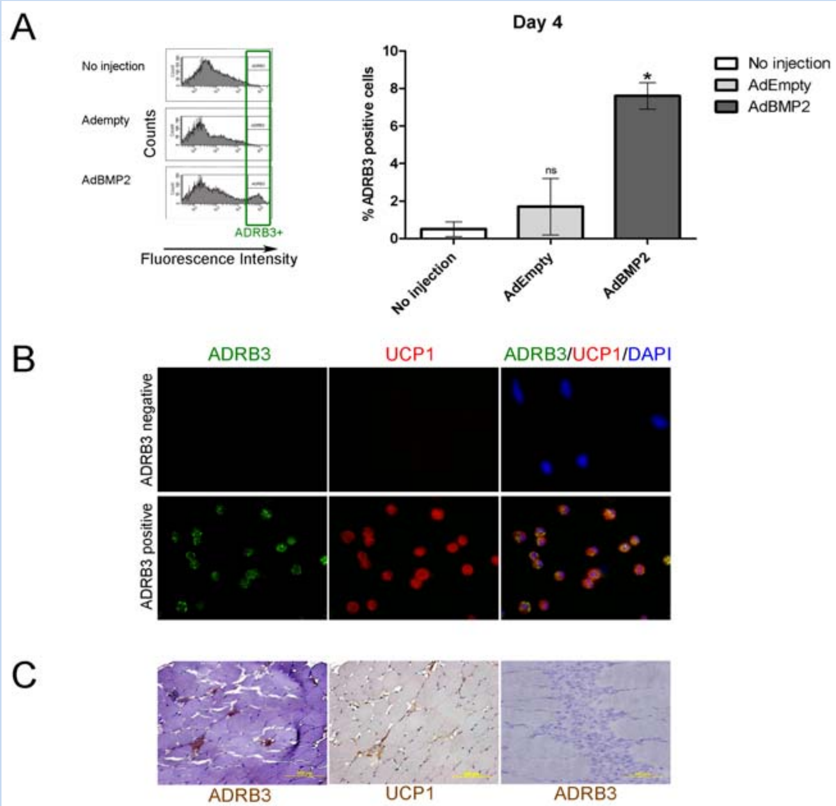


Figure 17: β adrenergic receptor 3 expression is associated with brown adipose in a model of heterotopic ossification. **A.**FACs analysis of ADRB3 immuno-staining of cells isolated from tissues surrounding the site of new bone formation, four days after induction. There was a significant difference between the tissues undergoing bone formation, but there was no significant difference between the tissues receiving Adempty cassette as compared to the normal tissues. Statistical significance was determined by a student t-test. **B.** ADBR3 positive and negative populations of cells isolated from the tissues surrounding the area of new bone formation, were obtained through cell sorting and immuno-stained for the brown adipose factor uncoupling protein 1 (UCP1). As seen in panel B, all UCP1 protein expression was found in the ADBR3 positive cell population. Green color represents positive staining for ADBR3 and red staining represents positive staining for UCP1. Cells were visualized by inclusion of the nuclear stain (DAPI, blue color). **C.** Co-immunostaining of UCP1 and ADBR3 cellular expression on serial sections in tissues surrounding the site of new bone formation four days after induction of bone. Tissues 20X, and cells 40X.

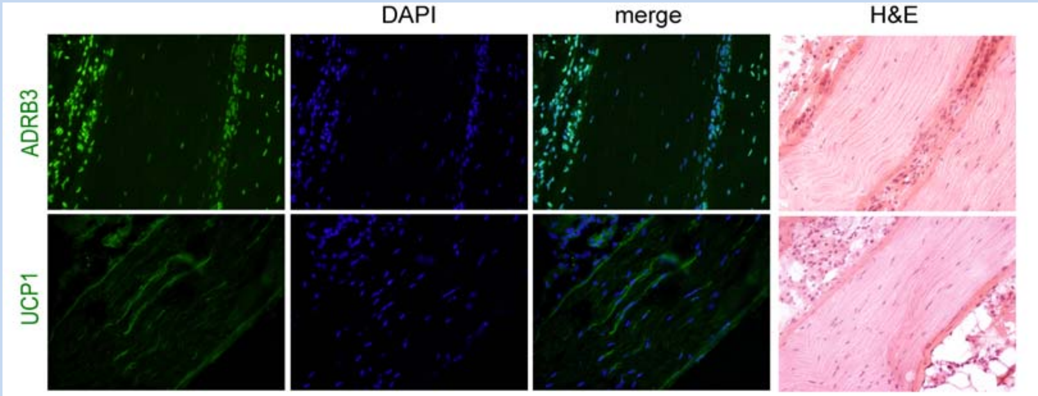


Figure 18: Photomicrograph of nerve tissues surrounding new bone formation two days after induction. Immunofluorescence staining for ADBR3 and UCP1 green color in cells associated with the nerve. Cells were visualized with DAPI (blue color).

We next looked at the expression of ADBR3 within the peripheral nerves, and found that the majority was associated with the epineurial adipose regions of the nerve (data not shown). Interestingly, immediately (48 hrs) after induction of new bone formation, the majority of ADBR3 cells appeared to be associated as before with the epineural region, however, now there was the appearance of cells within the perineural space. Immuno-staining for UCP1 showed that the majority of the cells within the epineural region were negative for UCP1, only the perinerual cells positive for ADBR3 appeared to co-express the UCP1, suggesting that they may be derived from a progenitor housed wihtin the nerve sheath (fig 18). We further performed FACs analysis for ADBR3 positive cells from isolated sciatic nerves after induction of new bone formation and found a statistically significant increase in nerves isolated from animals treated with BMP2 within 48 hours, but a statistically significant decrease in this same population in nerves which were isolated 4 days after induction, suggesting that the cells associated with the nerve may have migrated into the tissues for new bone formation. Further support of this, is finding that there is no statistically significant difference in ADRB3 cell populations within the tissues surrounding the site of new bone, until 3 days post induction, with the peak at 4 days, suggesting that these cells may have migrated from the nerve to the new location within

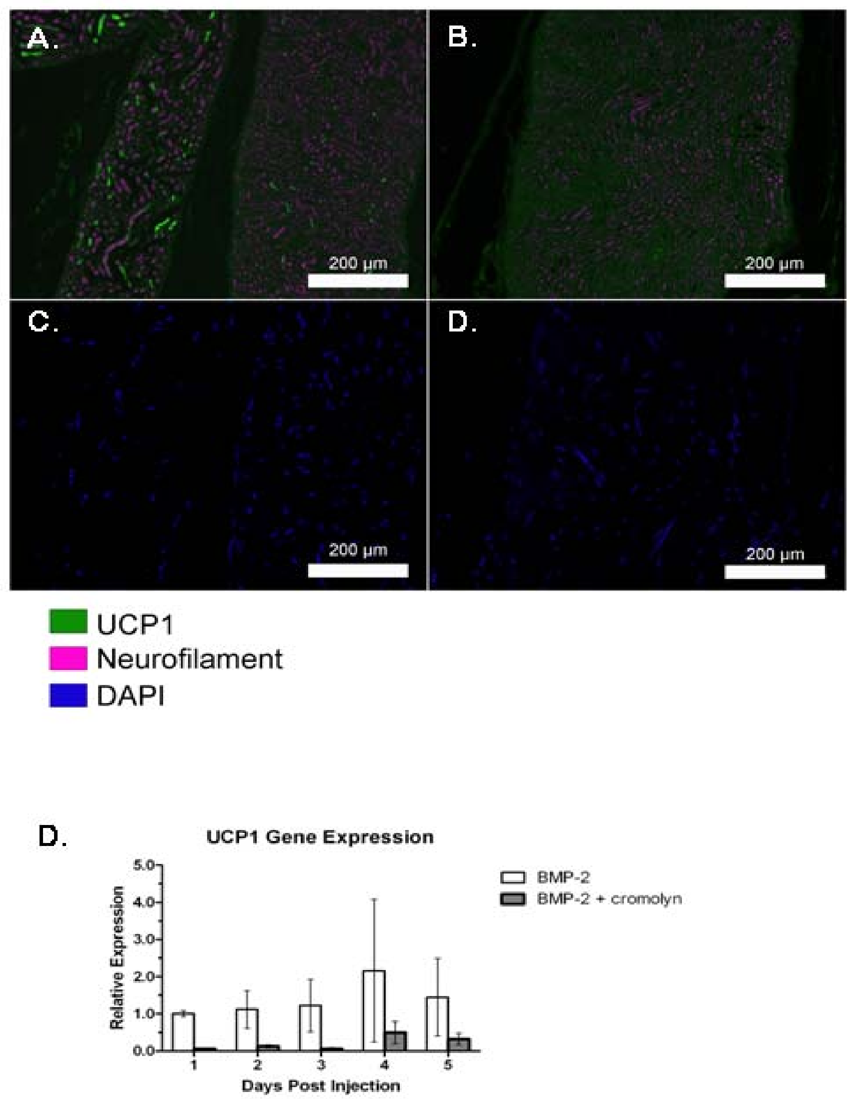


Figure 19. Photomicrographs of nerve tissues isolated 4 days after induction of new bone. **A,C:** without additional of cromolyn **B,D:** plus cromolyn. Quantitative RT-PCR for expression of UCP1 gene, in the presence and absence of cromolyn after induction of *de novo* bone formation.

the region of new bone formation (fig 17).
Since blocking mast cell degranulation stops local release of serotonin, we next looked at whether this would also block the production of brown adipose. Surprisingly, addition of cromolyn totally suppressed the expression of UCP1 RNA and protein within the nerve and surrounding tissues (fig 19). As can be seen in figure 19, the UCP1 positive staining nerves in the region of new bone formation 4 days after induction was absent in animals pretreated with cromolyn.

Additionally, we observed changes in the peripheral nerve after delivery of BMP2, with an increase in cells expressing early stem cell factors Klf 4 and Nanog in the presence of cromolyn. We hypothesize that the buildup in this potential progenitor cell is in part due to the inability of the cells to migrate from the nerve. We next questioned whether these cells could be contributing directly to the osteoblast populations so we

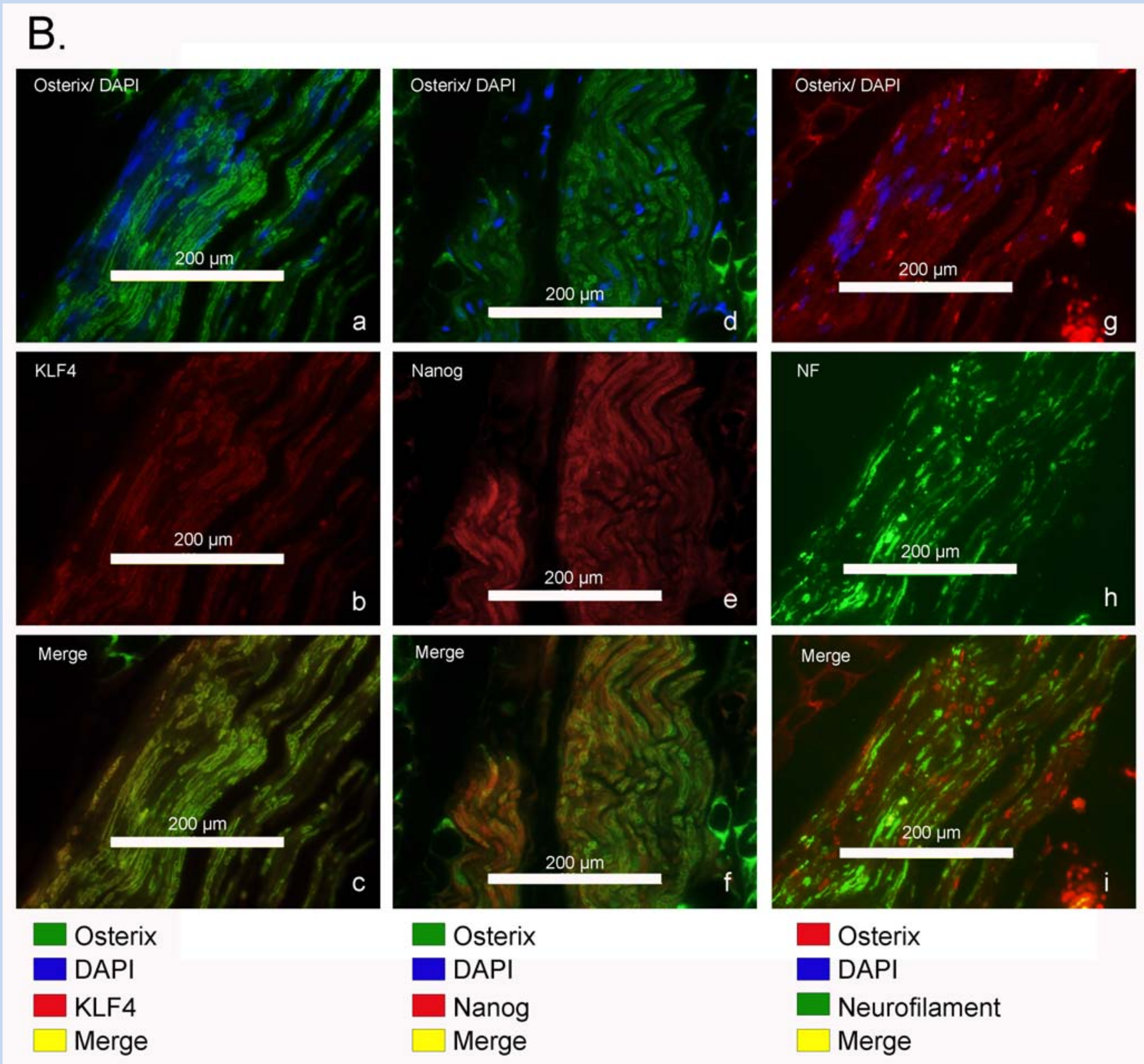


Figure 20: Microcomputational analysis of heterotopic ossification ten days after induction with AdBMP2 transduced cells, in mice pre-treated with either cromolyn or vehicle control (PBS). **(A)** Three dimensional reconstructions of representative samples for each group. **(B)** Quantification of bone volume and statistically significant changes between the groups was determined using a one-way analysis of variance; n=9. * denotes statistical significance.

immunostained with the osteoblast transcription factor, osterix. As seen in Fig 20, these nerve cells appeared to express osterix, suggesting that perhaps a subset of osteoblasts may be derived from the nerve. Additionally we also observed the presence of UCP1 positive staining in a subset of these cells suggesting that the brown adipocytes may also be derived transiently from the nerve (data not shown). Interestingly, mice deficient in dock 7, a GTPase shown to be selectively expressed in neural stem cells during migration, cannot produce brown fat [12]. Dock 7 has also been shown to be involved in the regulation of breast cancer cell migration [13]. The data collectively have led us to develop a mechanism by which the nerve regulates tissue regeneration (Fig 20). Based on our model of bone formation, we propose that loss of Nf1 function, disrupts the expansion and migration of the these nerve progenitors, ultimately forming tumors, rather than undergoing additional differentiation to the cell types necessary for tissue growth and maintenance.

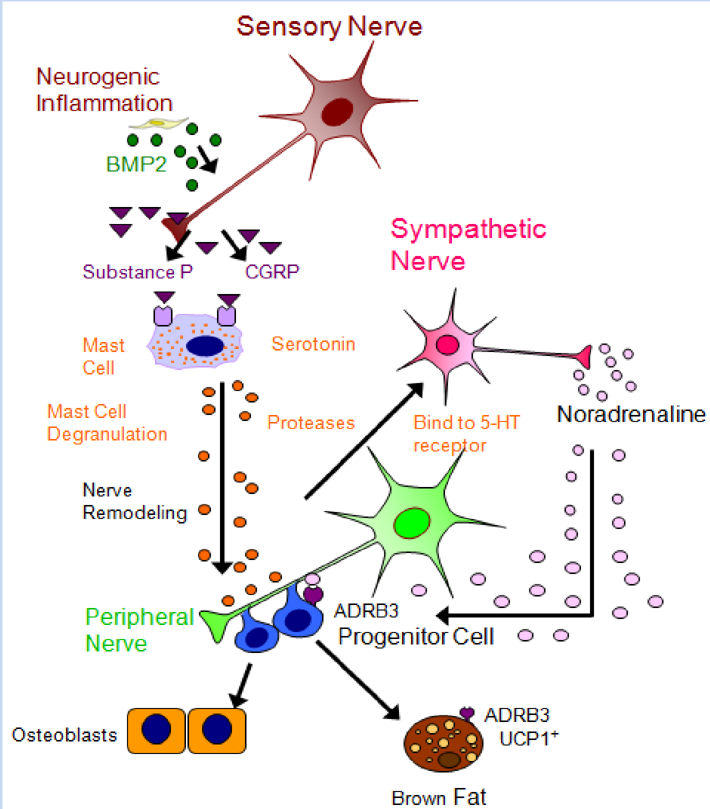


Figure 21. A model of nerve remodeling during early bone formation.

Task 2: Initiate preclinical efficacy testing of agents known to target and inhibit key molecules in HO.

1. Inhibition of neuroinflammatory molecules:

Our model suggests that there are two independent stages which may effectively target HO (figure 21). First we are testing the (1) substance P (SP) inhibitor CP-96,345 which noncompetitively inhibits this protein by binding at the neurokinin1 (NK1) site and has been widely used for this purpose. Therefore, approximately 1 nmole is injected intrathecally or into the spinal cord region in rats, every day for 1 week (n=16 per group) starting at the time of injection of the AdBMP2 or Adempty cassette transduced cells or similar animals which did not receive drug (n=10) animals are euthanized after two weeks, and bone quantified by microCT. The tissues are then isolated for histological analysis of the bone formation.

Inhibition of HO will be evaluated for all drug studies through microCT analysis. Statistical analysis to determine significance will be performed using SAS software version 9.1 (SAS Institute Inc., Cary, NC). The distribution data will be analyzed using the one-way ANOVA and/or Student's t-test. Correlations will be analyzed using Pearson correlation coefficients. The variables of imaging data and other assays will be considered correlated if the absolute r value is greater than 0.5 and the P value is less than 0.05.

2. Inhibition of heterotopic ossification by cromolyn through blocking mast cell degranulation

Since drugs already exist that can readily block mast cell degranulation, we next chose to look at whether inhibition of this step could affect HO in mouse models. We delivered the drug cromolyn or PBS, to mice prior to inducing HO, and results were compared to animals which did not receive the drug. The resultant microCT quantification (Fig 12) shows a significant decrease in HO in the presence of the drug. This preliminary study is based on one formulation of the drug and one dosage regime. We next immunostained the tissues for the presence of chymase and found the expression to be negative and similar to the control in the nerves. Since many studies in the literature suggest efficacy and bioavailability may be somewhat problematic with this agent, it is not surprising that we did not achieve total ablation. We are currently optimizing testing the different formulations of this compound to determine if we can optimize the inhibition of HO. These experiments involved the delivery of Gastrocrom, Oral Suspension (Celltech) which can be given orally up to 40 mg/kg/dose. This formulation is supposed to be more stable than the cromolyn we were injecting (i.p.) in our preliminary study. Thus it has been reported to be considerably more effective at blocking mast cell degranulation. **We have an ongoing study in both mice and Wistar rats using this drug to determine**

efficacy. We are performing the initial testing at the above dose, but upon the results will perform dose escalation studies, for optimization.

3. Inhibition of heterotopic ossification by AMD3100 through blocking stem cell extravasation: AMD3100 has been shown to be an efficacious antagonist of SDF1 and is available for testingin our model. AMD 3100, has currently gained FDA approval for inhibition of stem cell binding to CXCR4, resulting in mobilization of bone marrow stem cells. New data in the literature suggests that it also blocks stem cell entry into the targeted tissues when delivered locally. We are currently testing localized delivery of AMD3100 through i.m. injection into the site of bone formation. In these studies we inject the drug or PBS from 0-7 days after delivery of the AdBMP2 transduced cells and collect tissues, two weeks later for microCT. A series of mice were also euthanized and tissues collected starting 2-8 days after induction of bone formation at 24 hour intervals after delivery of the drug. We are in the process of analyzing these tissues. HO will be quantified to determine if the drug can reduce the overall formation of bone, but tissues will be analyzed for changes in tissue architecture, stages in formation, or recruitment of cells. Further, the drug will be delivered to a subset of the transplanted animals to determine if we can block cells possessing the reporter from entry. The deliver time will be varied to determine if we can specifically nerve, block cartilage and/or bone progenitors,independent of one another, and to determine the long term effect of this selective blockade. This will provide efficacy data based on the stages of HO and timing of delivery of the drug. These studies are being done in mouse models, so that we can use immunohistochemical approaches to analyze the changes, as well as the transplantation model. However, in depending on the effects on HO, we will also set up these experiments in Wistar rats, and confirm a similar effect on HO in a second model system.

Finally the last experiments that we are proposing to do, is to combine the potential inhibitors to determine if we see complete ablation when targeting multiple processes in HO.

Task 3: Complete preclinical testing with promising agents, and demonstrate specific targeting through toxicology.

With the recent data which shows that two targets appear to greatly reduce or ablate heterotopic ossification are as stated in the above sections (AMD3100 and cromalyn). These drugs are both clinically used, and are FDA approved. Thus toxicity is not as an issue, but rather, to obtain the optimal dose that will provide for the best effect. As discussed in the previous section, this will require dose escalation studies which are ongoing, formulation studies, to determine the optimal delivery route, and dosing regimen, that will provide the greatest efficacy. All ongoing studies as outlined in Task 2.

Therefore, in this aim we request permission to continue studies to develop a diagnostic which could detect these early stages. Statistically, heterotopic ossification associated with traumatic injury can appear anytime within a one year period of the injury. Since, neither drug is currently approved for chronic delivery, we propose to look for molecules associated with this early phase that could be developed as a diagnostic test for HO, and ultimately allow us to determine when to deliver the potential drugs to the individuals. Further, depending on the results of Task 2, we may also find that local delivery of the compounds may be more effective than systemic. Thus having the ability to rapidly detect the involved region, would provide potentially greater efficacy to these drugs.

Our proposed plan for developing a diagnostic is based on our tentative model system in which we observed small but statistically significant changes in blood platelet levels. We propose to also look at the level of CXCL7 in the blood of these animals. In humans, CXCL7 is diagnostic for myelodysplastic disease [14], and current rapid blood serum tests have been developed to detect this factor in peripheral blood. We will confirm in our mouse model, that we see a similar associated increase in CXCL7 within the blood after induction of HO. We envision that the initial diagnostic screen would potentially be simple blood work done on a regular basis for up to the first year after traumatic injury. Upon changes in blood chemistry, we envision a second diagnostic screen, which could not only confirm the induction of HO, but potentially localize it for drug delivery.

a. Develop novel methods to detect early HO in vivo.

To better track both the bone formation and potential inhibition by selective agents *in vivo* we investigated developing an imaging modality that could selectively track specific molecules known to be involved in the earliest stages of bone formation. To this end have developed an imaging modality for the detection of new heterotopic bone formation that targets activated MMP9 and thus can detect the early nerve remodeling prior to matrix production. We have recently published these findings in the journal Tissue Engineering. To confirm that the elevation in MMP-9 RNA expression translated into enhanced protein expression in the tissues, MMP-9 protein was quantified by ELISA. Protein extracts were generated from the tissues isolated at daily intervals starting 48 hours after delivery of the transduced cells, and again ending at the onset of bone matrix production. Figure 3A shows changes in total MMP-9 protein over the course of heterotopic ossification. MMP-9 protein appears to be significantly ($p< 0.05$) elevated within 24 hours after delivery of the transduced cells regardless of the presence or absence of BMP2, but this elevation dropped rapidly, in the control tissues, whereas the tissues undergoing heterotopic ossification remained elevated. As seen in figure 3A, MMP-9 total protein was significantly higher in the BMP2-exposed tissues as compared to those receiving the control transduced cells with the exception of day 3. This may in part be due to the variability within the samples isolated from control tissues, and in part to the downward trend in total protein, after day 2. Interestingly, the level of MMP-9 protein trends upward again starting with day 4, attaining levels similar to those observed on day 2, at the time where cartilage matrix is undergoing degradation, and replacement with bone (day 6) (figure 3A).

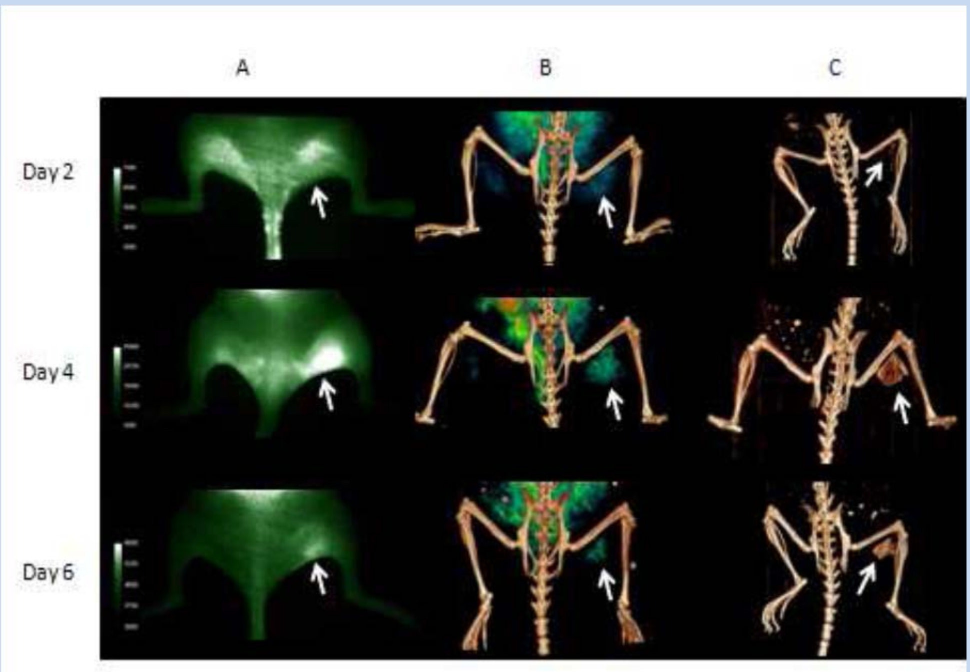


Figure 5. µPET/CT imaging of new bone formation with ^{64}Cu-M$_2$. Panel A shows the fused µPET/CT image at day 4 post-implantation (left), the day 4 µCT alone (center) and the day 11 CT. Panel B shows the fused µPET/CT image from the blocking study.

Since our current peptide reagent preferentially detects active MMP-9, we next chose to measure amounts of MMP-9 using an ELISA-based system that detects only active MMP9. The results (Fig. 3B) show that protein extracts isolated from tissues receiving the BMP2-producing cells have significantly more active MMP-9 protein, than those receiving control cells and these elevated levels remained unchanged across the course of HO. The results collectively suggest that MMP-9 is activated by delivery of the BMP2-producing cells, during all stages of endochondral bone formation. Further, this activation may be due to cleavage and utilization of stored MMP-9 protein, immediately following induction of HO, but is then rapidly replaced by newly synthesized MMP-9. Activated MMP-2 remained below the level of detection (data not shown).

We next looked at the expression patterns of MMP-9 within the tissues undergoing HO or the control using fluoresence microscopy. Interestingly, MMP-9 (red staining) was observed in tissues isolated 24 and 48 hours after delivery of AdBMP2 and Adempty transduced cells (figure 4). However by 72 hours, we were unable to detect MMP-9 positive cells within the tissues receiving Adempty (data not shown). MMP-9 expression (red) appeared to be associated with the nerve tissues (green staining) in the tissues one day after receiving AdBMP2 transduced cells, whereas MMP9 positive cells appeared to be uniformly dispersed within the control tissues (figure 4). In limbs injected with BMP2, at day two, we observed both nerve associated expression similar to day 1 (Figure 4), but also localization near von Willibrand factor (VWF) positive vasculature (yellow staining). This pattern continued through day four just prior to the appearance of cartilage (figure 1 and 4). Presumably this expression is associated with remodeling of the tissues at a point when progenitors are assembling to form the initial cartilage condensation. The timing of MMP-9 expression within the tissue appears to match those predicted by the RNA and protein analysis. A patent has been filed for this technology.

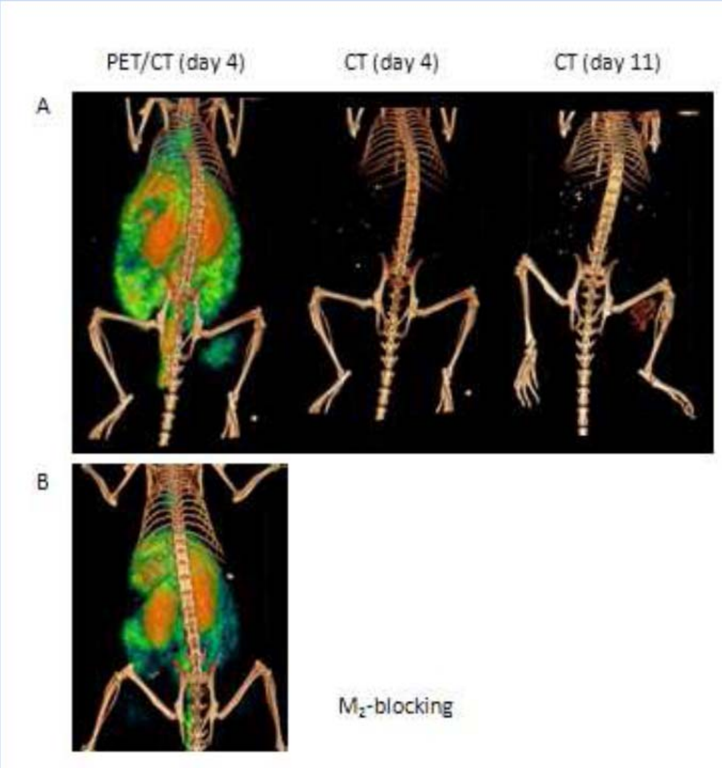


Figure 6. Multimodality imaging of new bone formation with ^{64}Cu-M_2. NIR fluorescence (A) and μPET/CT and images (B) were acquired on days 2, 4 and 6 post-implantation. The follow-up CT scans (C) were performed on day 9, 11 and 13 post-implantation to confirm the presence of HO and correlate with agent uptake observed by molecular imaging.

A. Screen compounds for testing
1. Inhibition of heterotopic bone formation by CXCR4 through AMD3100

We have not pursued this since our current studies show that cromolyn is extremely effective at blocking heterotopic ossification, and has a significant safety record for clinical use. Recently, our findings have been confirmed through studies of Kan *et al*[15], which also shows the potential for targeting the early neurogenic inflammation in patients with spinal cord injury that are at high risk for HO. Several compounds are available at potential stages of drug development that would be efficacious for prevention of HO, if delivered early enough. **Thus we propose that cytokine changes within the blood stream as well as changes in blood platelet levels could be potential early biomarkers of this process, and could be rapidly confirmed using our imaging modality. Once confirmed doctors could then potentially provide either local delivery of the compounds or systemic. From completion of these studies, we are poised to initiate clinical studies with additional funding, that would allow us to test both the imaging modality and the biomarker, as well as test approved drugs such as cromolyn for prevention of HO.**

Key Research Accomplishments:

- We have identified the earliest stages of heterotopic ossification.
- We have demonstrated role for sensory neurons in the induction of heterotopic ossification
- We have identified a tentative molecular mechanism of how BMP2 and/or traumatic injury can lead to heterotopic ossification
- We have the induction of new vessel formation, which is regulated by brown adipose.
- We have observed a number of inflammatory associated cells entering the tissues through extravastion.
- We have identified a unique nerve progenitor/stem cell, which contributes to heterotopic ossification.
- We have published recent manuscript on this work (see next section)
- We have been asked to present these findings at both national and international meetings, and presented the topic through a review article requested by the by past president of the American Society of Bone and Mineral Research.
- We have been asked to present these findings at the annual ASBMR meeting.
- We have developed a method for optical- microPET detection of heterotopic ossification prior to matrix deposition.
- We have demonstrated inhibition of HO through regulation of sensory neuron activity in TRVP1 knockout mice.

- We have demonstrated inhibition of heterotopic ossification through systemic delivery of cromolyn.
- We have noted neuronal remodeling, and neurite outgrowth towards the new bone formation (publication in preparation)
- We have shown elevation early on of blood platelets in the mice, which may be useful as a biomarker for early HO.
- We have developed a mechanism for early diagnosis of HO prior to matrix deposition that could be used as an initial screen for to detect this process.
- We have applied for a patented on this technology.

Reportable Outcomes:
Reportable Outcomes:
Oral presentation:
Sonnet C, Rodenberg EJ, Salisbury EA, Olmsted-Davis EA, Davis AR. A model for neuronal regulation of heterotopic ossification. Conference: 7th International Society of Musculoskeletal and Neuronal Interactions (ISMNI). Cologne, Germany, May 2010. Presenter - Corinne Sonnet.

Olmsted-Davis E. A., Davis, A.R. and West, J. L. The Role of the Peripheral Nervous System in Heterotopic Ossification. Advances in Mineral Metabolism and John Haddad Young Investigators Meeting/ASBMR, Aspen, CO April 6-10.

Olmsted-Davis, et al. Osteoinductive microspheres. Bone Disease Program of Texas, Houston, TX March 2nd, 2011.

Poster presentation:
E. Salisbury, Z. Lazard, E. Rodenberg, A.R. Davis, and E.A. Olmsted-Davis. Neuronal Regulation of Early Heterotopic Ossification. Texas Bone Program Annual Meeting, May 10, 2010. Houston TX.

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Pending patents:

Patent: U.S. Provisional Application No.: 61/483,600, "Methods for Imaging Bone Precursor Cells Using Dual-Labeled Imaging Agents to Detect MMP-9 Positive Cells", invented by Alan R. Davis, *et. al.* Filed: May 6, 2011.

International Patent Application No.: PCT/US2010/58603
Title: Methods and Compositions for Bone Formation
Filling Date: December 1, 2010
Inventor: Elizabeth A. Davis, *et al*

Conclusions:

We have made significant progress for this final of the award. We hope to translate these findings into the clinic in subsequent potential grant awards. We have identified a novel model and greatly enhanced our understanding of heterotopic ossification. Further we have completed the initial studies to provide the tools necessary for early detection, and intervention, for its treatment. Not only have we described this in our system, others have now confirmed our findings in their own models of heterotopic ossification, suggesting that this is a paradigm shifting finding. Moreover we have confirmed the efficacy of cromolyn in rodent models, and are poised to translate both the diagnostic and drug into the clinics.

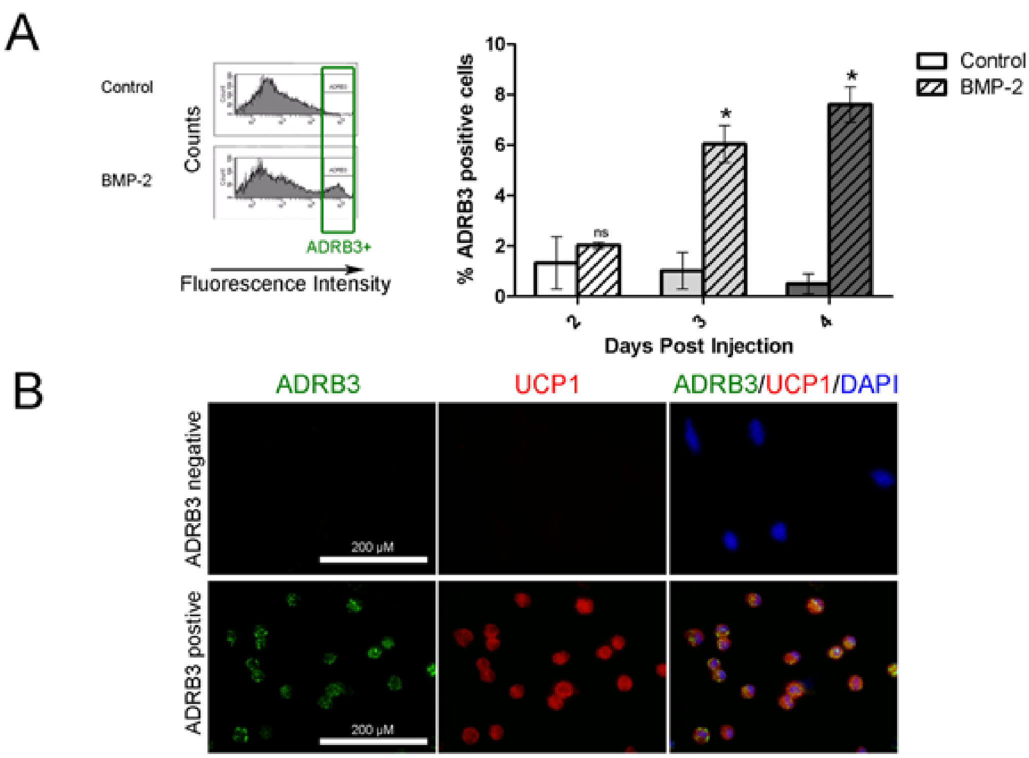
Finally, it should be noted that the work performed under this grant represents a major change from what is currently known in bone biology. It is now apparent to us that peripheral nerves indeed house primitive progenitors that are likely neural crest stem cells. These cells are not only important in heterotopic ossification, but also in bone repair as well as bone homeostasis. It also appears likely to us that mutations and defects in these stem cells and the process of neurogenic inflammation by which they are released from the nerve are the basis of many diseases. One can see that diseases such as Ewing's sarcoma, neurofibromatosis, and tuberous sclerosis, which involve bone defects as well as tumors, are obvious extensions of this reasoning. However, it is not unlikely that major cancers, including breast and prostate, have their basis within these cells because these tissues, like bone, are controlled by sex steroids and have major changes in the adult during which these tissues signal to nerve their need for them. Also, this type of signal, from the tissues themselves for new progenitors for developmental changes, may also play a major in the diseases themselves.

No cost extension: Transient brown adipogenesis

We have utilized the period of the no cost extension to characterize and define a very novel molecular target in HO: transient brown fat. We have found that: **1.** Peripheral nerves house progenitors for brown adipocytes **2.** Upon BMP2 treatment these progenitors are activated by virtue of norephinephrine binding to β3 adrenergic receptors (ADRB3) present on their surface and begin egress from the peripheral nerve **3.** Upon exit from the nerve these cells begin to replicate and express UCP1 **4** Transient brown adipocytes are a major controller of **a.** micorenvironmental oxygen tension by virtue of UCP1 mediated oxygen burning[1] **b.** neovascularization and new lymphangiogenesis by virtue of their secretion of VEGFA and D[16] **c**. neurogenesis by virtue of their production and secretion of reelin. Although points a and b have been previously published by us, the production of reelin by tBAT is indeed new and very novel. We have summarized the data for this below and note that the original description of reelin production in the brain by Cajal-Retzius cells does not rule out the possibility that these cells are also tBAT. This is due to the fact that brown fat has been found to play a significant role in Huntington's disease, which is known to affect the brain[17].

Our preliminary data suggests that one of the first steps in HO is the production of transient brown adipose tissue (tBAT)[1]. We have now determined that tBAT arises from ADBR3+ cells within the epineurial region of peripheral nerves, which expand and migrate towards BMP2 or the site of new bone formation. Fluorescence activated cell sorting of ADBR3+ cells followed by cytospin, and immunocytochemistry revealed that 100% of UCP1 expression was associated with this population (Figure 1). A similar rapid formation of these cells occurs early in fracture repair. Using a mouse fracture repair model where a cortical defect is made in the tibia[18], we observed the presence of UCP1+ tBAT in the periosteum of the fractured but not contralateral bone as early as two days after injury (Figure 2), suggesting that tBAT may also serve a critical function in fracture repair.

Figure 1: Isolation of tBAT using the ADBR3 surface marker.



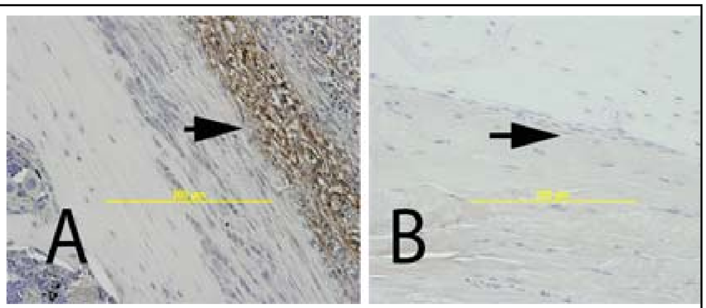


Figure 2. UCP1 staining in the periosteum of a fractured mouse tibia 2 days after injury. A mouse tibia was fractured (A) and the contralateral tibia was untreated (B). The mouse was euthanized and paraffin sections prepared after decalcification. Sections were stained for UCP1. The fracture is just below the image in panel A. Arrows, periosteum.

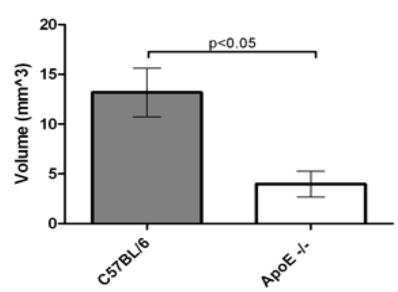


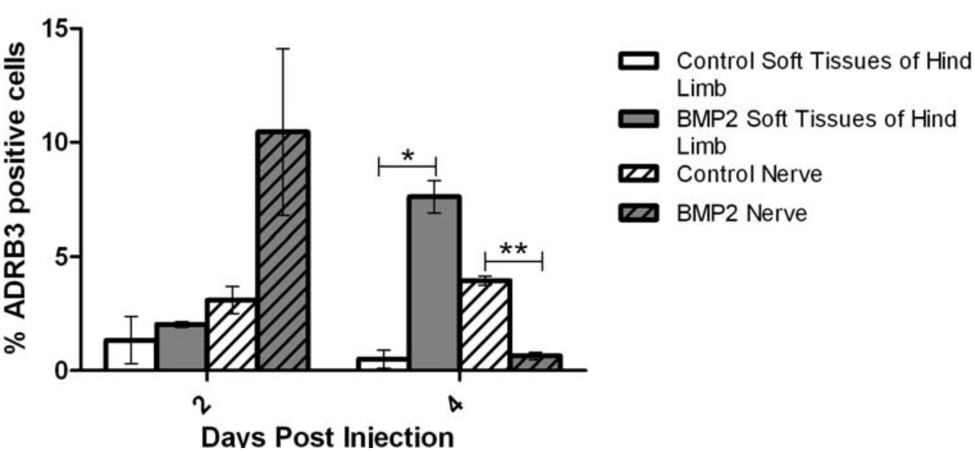
Figure 3: Quantification of bone volume in Apo E-/- mice or wild type counterpart using microCT analysis. n=10. Significance was determined using a standard T-test.

BAT is a unique form of adipose tissue induced by cold exposure[19], which has recently been shown to be present in adults[20]. One of the unique features of BAT is the uncoupling of the electron transport chain during aerobic respiration leading to the release of the energy as heat, rather than ATP. Although the release of heat by BAT has been the focus of studies on non-shivering thermogenesis, even simple heating can be a mechanism for generation of hypoxia since the solubility of oxygen in water decreases with increasing temperature. However, it now seems clear that BAT found in depots[21, 22], is not the same as tBAT [23 24]. Here, we hypothesize a different potential functional role for tBAT in bone formation. We propose that tBAT functionally contributes to this process through regulation of oxygen pathways essential for expansion and recruitment of progenitors. Further we hypothesize that tBAT enables this functionality by forming gradients of oxygen tension through uncoupled aerobic respiration intimately coupled with the hypoxia inducible factor (HIF1) and pathways under its control. We have shown that animals lacking the ability to generate tBAT surprisingly made more heterotopic bone[1]. However, in these studies white adipose tissue (WAT) appeared to be uniquely hypoxic, suggesting that it may compensate for the lack of tBAT through increased respiration[1] Others have shown that WAT can compensate or be converted to brown-like adipose[24]. Since respiration, either uncoupled or not, requires oxygen and triglycerides to support electron transport, we reasoned that blocking triglyceride uptake by adipocytes could effectively suppress respiration. Recently, Bartelt *et al* showed that BAT activity controls triglyceride clearance[25]. Triglyceride uptake and storage in adipocytes is achieved through ApoE-directed phospholipid conversion to low density lipoprotein complexes, which then bind to either VLDLR or ApoER2 receptors for cellular uptake. Studies have shown that in the absence of ApoE, adipocytes cannot replenish their triglyceride stores[26 27, 28]. We analyzed HO in a mouse Apo $E^{-/-}$ mouse model (fed normal diet) and found a significant suppression and in many cases complete ablation of HO (n=10). In these studies HO progression appeared to be suppressed or in many cases totally ablated (Figure 3).

Progenitors for tBAT arise from the epineurium of the peripheral nerve

We have used the kinetics of synthesis of tBAT after BMP2 induction to clearly show that it arises from progenitors in the nerve that leave the nerve and then differentiate into tBAT. Figure 4 shows that in the peripheral nerve itself, ADRB3 cells are high on day 2 and low on day 4. This is in contrast to the ADRB3 positive cells outside of the nerve (in muscle tissue), which are low on day 2 and high on day 4. The most reasonable explanation of this result is that progenitors in the nerve exit the nerve and are found in the adjacent tissue at later times

Figure 4. Kinetics of accumulation of ADRB3 cells within and outside of peripheral nerves after BMP2 induction.



Transient brown fat appears and disappears rapidly and seems not to utilize many of the mechanism known to occur in interscapular brown fat.

In Figure 5 below we show analysis of the time course of synthesis of key mRNAs for tBAT as assessed by quantitative reverse transcriptase PCR. Particularly noteworthy is a peak of UCP1 mRNA, increasing 70 fold on day 3 while on day 4 dropping to a relative increase of less than 5 fold. However, the mRNAs of PPARG, PPARA, and PRDM16 do not change during the course of BMP2 induction (not shown), even though these molecules are known to be critical in the biogenesis of interscapular brown adipocytes.

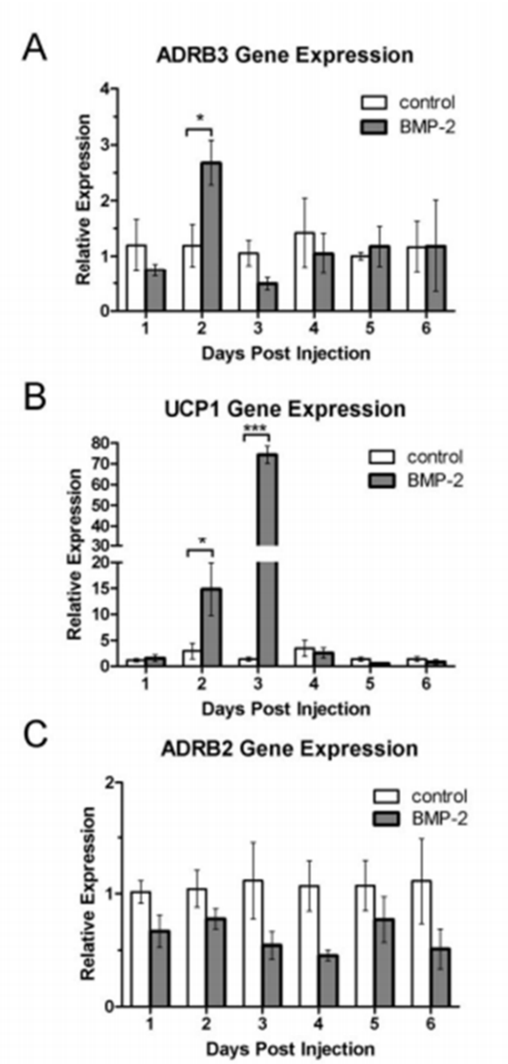


Figure 5. Kinetics of RNAs specific for A, ADRB3; B, UCP1; and C, ADRB2 during BMP2 induction

Transient brown fat expresses reelin

The experiment shown in Figure 3 above indicates that when BMP2 induction is performed in an $ApoE^{-/-}$ mouse that very little bone is formed. Other preliminary data (not shown) indicates that BMP2 also initiates neurogenesis in this model. We therefore tested tBAT for the expression of reelin since reelin and ApoE utilize the same receptors and ApoE interferes with reelin binding. Accordingly $ADRB3^{+}$ cells were isolated by FACS 3 days after BMP2 induction and the cells centrifuged onto a microscope slide (cytospin). This cytospin was then probed with antibodies against reelin, ADRB3, UCP1 as well as stained with DAPI. As can be seen in Figure 6 below, ADRB3 positive cells express UCP1 as expected, but they also express reelin.

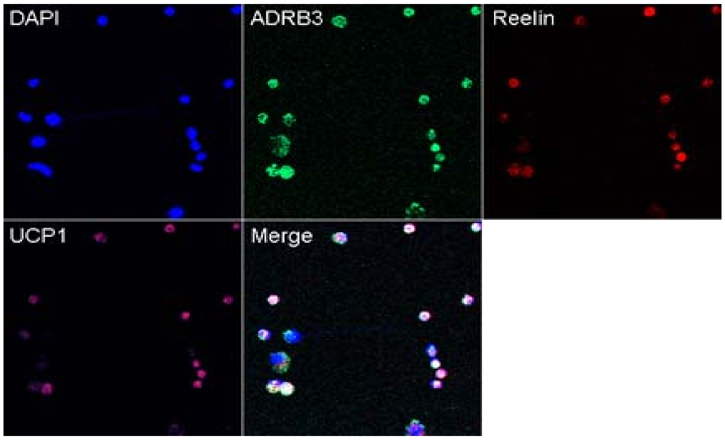


Figure 6. Expression of reelin by tBAT. ADRB3$^+$ cells were isolated by cell sorting, subjected to cytospin onto a microscope slide, stained with DAPI, and probed with antibodies against ADRB3, reelin, and UCP1.

The fact that tBAT produces reelin means that tBAT is a key cellular entity in tissue programing by BMP2. Transient brown adipocytes therefore have the ability to initiate and control neurogenesis, neovascularization, as well as cartilage and bone formation. It is therefore not surprising that this tissue plays major roles in bone formation and repair and has recently been shown to also play a major role in bone homeostasis[29].

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